09/24/102 ON - LINE TEXT VALIDATION PAGE 1

14:04:56 E R R O R R E P O R T

PATENT NO: 56328511.001 GROUP: T1

ISSUE DATE: 08/05/02

PAGE NO: 5 LINE NO: 127 WARNING => MISSING LIGHT COMMAND (+l) FOLLOWING (+b) AFTER 50 !!

PAGE NO: 28 LINE NO: 812 WARNING => MISSING LIGHT COMMAND (+l) FOLLOWING (+i) AFTER 50 !!

PAGE NO: 28 LINE NO: 820 WARNING => MISSING LIGHT COMMAND (+l) FOLLOWING (+i) AFTER 50 !!

D A C S - E R R O R R E P O R T

PATENT #: 56328511.001 ISSUE DATE: 08/05/02 GROUP: T1

ERROR, Found Alpha Char Inside +B Command.
ERROR, Page #: 47, Line #: 1251

INFO, +E,FRA (Fraction Cmd) - Total Commands Found: 2

INFO, Total Invalid Plus Commands Found: 00001
INFO, Total ALL Text DACS Validation Errors: 00001
INFO, Total ALL Text DACS Validation Warnings: 00000

ENDED TEXT DACS VALIDATION FOR: ***** 56328511.001 *****

--* NEW PATENT *-*-*

Group T1

PATENT # 56328511.001

0001 +pg,1

0002 +sa

0003 The present invention generally relates to methods of making cDNA

0004 molecules and cDNA libraries. The invention also relates to cDNA <<<<

>>>>molecules

0005 and cDNA libraries produced according to these methods, as well as to <<<<

>>>>vectors

0006 and host cells containing such cDNA molecules and libraries. The <<<<

>>>>invention

0007 also relates to kits for making the cDNA molecules and libraries of the

0008 invention.

0009 +ea

0010 +pg,2

0011 +sp +cl CROSS REFERENCE TO RELATED APPLICATIONS

0012 +p This application claims the benefit, under +b 35 +l U.S.C. +517 +0 <<<<
>>>>+b 119+l (e), of U.S.

0013 Provisional Application No. +b 60/122,395, +l filed on Mar. +b 2, <<<<
>>>>1999, +l which is

0014 fully incorporated by reference herein.

0015 +su +cl BACKGROUND OF THE INVENTION

0016 +p The present invention relates to the field of molecular and cellular

0017 biology. The invention generally relates to methods of synthesizing cDNA.

0018 More specifically, the present invention relates to methods of <<<<
>>>>increasing the

0019 average cDNA insert size and more particularly, to increasing the <<<<
>>>>percentage

0020 of full-length cDNA present within cDNA libraries. Thus, the present

0021 invention provides improved cDNA libraries useful in gene discovery.

0022 +p In examining the structure and physiology of an organism, tissue or

0023 cell, it is often desirable to determine its genetic content. The genetic

0024 framework of an organism is encoded in the double-stranded sequence of

0025 nucleotide bases in the deoxyribonucleic acid (DNA) which is <<<<
>>>>contained in the

0026 somatic and germ cells of the organism. The genetic content of a <<<<
>>>>particular

0027 segment of DNA, or gene, is only manifested upon production of the <<<<
>>>>protein

0028 which the gene encodes. In order to produce a protein, a complementar<<<<
>>>>y copy

0029 of one strand of the DNA double helix (the +37 coding+38 +0 strand) <<<<
>>>>is produced by

0030 polymerase enzymes, resulting in a specific sequence of ribonucleic acid
0031 (RNA). This particular type of RNA, since it contains the genetic message
0032 from the DNA for production of a protein, is called messenger RNA (mRNA).
0033 +p Within a given cell, tissue or organism, there exist many mRNA
0034 species, each encoding a separate and specific protein. This fact <<<<
>>>>provides a
0035 powerful tool to investigators interested in studying genetic <<<<
>>>>expression in a

0036 +pg,3

0037 tissue or cell. mRNA molecules may be isolated and further manipulated by

0038 various molecular biological techniques, thereby allowing the <<<<

>>>>elucidation of

0039 the full functional genetic content of a cell, tissue or organism.

0040 +p A common approach to the study of gene expression is the production

0041 of complementary DNA (cDNA) clones. In this technique, the mRNA

0042 molecules from an organism are isolated from an extract of the cells <<<<

>>>>or tissues

0043 of the organism. This isolation often employs solid chromatography <<<<

>>>>matrices,

0044 such as cellulose or agarose, to which oligomers of thymidine (T) <<<<

>>>>have been

0045 complexed. Since the +b 3+l +40 +0 termini on most eukaryotic mRNA <<<<

>>>>molecules contain

0046 a string of adenosine (A) bases, and since A binds to T, the mRNA <<<<

>>>>molecules

0047 can be rapidly purified from other molecules and substances in the <<<<

>>>>tissue or

0048 cell extract. From these purified mRNA molecules, cDNA copies may be

0049 made using the enzyme reverse transcriptase (RT) or DNA polymerases

0050 having RT activity, which results in the production of single-<<<<

>>>>stranded cDNA

0051 molecules. The single-stranded cDNAs may then be converted into a

0052 complete double-stranded DNA copy (i.e., a double-stranded cDNA) of the

0053 original mRNA (and thus of the original double-stranded DNA sequence,

0054 encoding this mRNA, contained in the genome of the organism) by the <<<<

>>>>action

0055 of a DNA polymerase. The protein-specific double-stranded cDNAs can then

0056 be inserted into a vector, which is then introduced into a host <<<<
>>>>bacterial, yeast,
0057 animal or plant cell, a process referred to as transformation or <<<<
>>>>transfection.
0058 The host cells are then grown in culture media, resulting in a <<<<
>>>>population of
0059 host cells containing (or in many cases, expressing) the gene of <<<<
>>>>interest or
0060 portions of the gene of interest.
0061 +p This entire process, from isolation of mRNA to insertion of the cDNA
0062 into a vector (e.g., plasmid, viral vector, cosmid, etc.) to growth <<<<
>>>>of host cell
0063 populations containing the isolated gene or gene portions, is termed <<<<
>>>>+37 cDNA
0064 cloning.+38 +0 If cDNAs are prepared from a number of different mRNAs,<<<<
>>>> the
0065 resulting set of cDNAs is called a +37 cDNA library,+38 +0 an <<<<
>>>>appropriate term since
0066 the set of cDNAs represents a +37 population+38 +0 of genes or <<<<
>>>>portions of genes

0067 +pg,4

0068 comprising the functional genetic information present in the source <<<<
>>>>cell, tissue

0069 or organism. Genotypic analysis of these cDNA libraries can yield much

0070 information on the structure and function of the organisms from which <<<<
>>>>they

0071 were derived.

0072 +p The ability to increase the total amount of cDNA produced, and more

0073 particularly to produce a cDNA libraries having an increase in the <<<<
>>>>average size

0074 of the cDNA molecules and/or to produce cDNA libraries having an increase

0075 in the percentage of full-length cDNA molecules would provide a <<<<
>>>>significant

0076 advance in cDNA library construction. Specifically, such advances would

0077 greatly improve the probability of finding full-length genes of interest.

0078 +p Ideally, synthesis of a cDNA molecule initiates at or near the +b <<<<
>>>>3+1 +40 +0 termini

0079 of the mRNA molecules. Priming of cDNA synthesis at the +b 3+1 +40 +0 <<<<
>>>>termini at the

0080 poly A tail using an oligo(dT) primer ensures that the +b 3+1 +40 +0 <<<<
>>>>message of the

0081 mRNAs will be represented in the cDNA molecules produced. Priming which

0082 occurs within the mRNA molecules (internal priming) results in <<<<
>>>>synthesis of

0083 cDNA molecules which do not contain the full-length message for the <<<<
>>>>genes of

0084 interest. That is, internal priming results in truncated cDNA <<<<
>>>>molecules which

0085 contain only a portion of the gene or genes of interest. Typically, <<<<

>>>>internal

priming causes a loss of the +b 3+l +40 +0 sequences from the message <<<<

>>>>population. Thus,

internal priming lowers the total amount of cDNA produced, decreases the

average insert size of cDNA molecules for a cDNA library and/or decreases

the percentage of full-length cDNA molecules in a given cDNA library.

Sequencing analysis has indicated that many eukaryotic mRNAs have <<<<

>>>>internal

poly adenylation stretches which may serve as a priming site when an

oligo(dT) primer is used for first strand cDNA synthesis with reverse

transcriptase. Moreover, research has shown that some mRNAs can have as

many as +b 16 +l internal priming sites (Lovett, M., et al., The <<<<

>>>>construction of full-length

cDNA libraries by conventional methods and a novel double capture

technique, University of Texas Southwestern Medical Center, Dallas, Tex.,

presented at the +b 48+hu th +l Annual Meeting held by The American <<<<

>>>>Society of Human

0098 +pg,5

0099 Genetics, Oct. +b 27+14 31, 1998, +l Denver, Colo.). Thus, internal <<<<
>>>>priming of

0100 the primer to such internal poly A sequences may adversely affect cDNA

0101 synthesis.

0102 +p The present invention alleviates, prevents, reduces or substantially

0103 reduces internal priming thereby providing improvements in cDNA and cDNA

0104 library construction. Accordingly, the present invention greatly <<<<
>>>>facilitates

0105 gene discovery by providing cDNA libraries containing a greater <<<<
>>>>percentage of

0106 full-length genes.

0107 +p The present invention therefore relates to synthesizing a cDNA

0108 molecule or molecules from an mRNA template or population of mRNA

0109 templates under conditions sufficient to increase the total amount of <<<<
>>>>cDNA

0110 produced, increase the length of the cDNA molecules produced, and/or

0111 increase the amount or percentage of full-length cDNA molecules produced.

0112 In accordance with the invention, any conditions which inhibit, prevent,

0113 reduce or substantially reduce internal priming may be used. Such <<<<
>>>>conditions

0114 preferably include but are not limited to optimizing primer <<<<
>>>>concentrations,

0115 optimizing reaction temperatures and/or optimizing primer length or

0116 specificity. Such result may also be accomplished in accordance with the

0117 invention by optimizing the reverse transcription reaction, preferably by

0118 inhibiting or preventing reverse transcription until optimum or <<<<
>>>>desired reaction

0119 conditions are achieved.

0120 +p Conventional methods for constructing cDNA libraries use a molar

0121 ratio of oligo(dT) primer/mRNA template of +b 15:1 +l for first <<<<

>>>>strand cDNA

0122 synthesis. The use of such excess amounts of oligo(dT) primer allows <<<<

>>>>internal

0123 priming of one or more primers to one or more of the mRNA templates <<<<

>>>>in the

0124 reaction. According to a preferred aspect of the present invention, <<<<

>>>>the amount

0125 of oligo(dT) primer is reduced for synthesis of first strand cDNA to <<<<

>>>>inhibit,

0126 prevent, reduce or substantially reduce internal priming. Preferred molar

0127 ratios of primer to template range from about +b 12:1; 10:1; 9:1; <<<<

>>>>8:1; 7:1; 6:1;

0128 5:1; 4:1; 3:1; 2:1; 1:1; 1:2; 1:3; 1:4; 1:5; 1:6; 1:7; 1:8; 1:9; 1:10 <<<<

>>>>+l and +b 1:12. +l

0129 +pg,6

0130 Preferably, molar ratios of primer (e.g., oligo(dT)) to template <<<<
>>>>(e.g., mRNA)

0131 range from about +b 5:1 +l to about +b 1:20, +l although lower molar <<<<
>>>>ratios of primer to

0132 template may be used in accordance with the invention. Specifically, <<<<
>>>>molar

0133 ratios of primer to template may be below about +b 1:10; 1:15; 1:20; <<<<
>>>>1:25; 1:50;

0134 1:75; +l and +b 1:100. +l Preferably, ranges of molar ratios are <<<<
>>>>below about +b 5:1; 4:1;

0135 3:1; 2:1; 1:1; 1:2; 1:3; 1:4; +l and +b 1:5. +l Most preferably, <<<<
>>>>ratios of primer to

0136 template range from about +b 10:1 +l to +b 1:10; 5:1 +l to +b 1:10; <<<<
>>>>4:1 +l to +b 1:10; 3:1 +l to +b 1:10;

0137 2.5:1 +l to +b 1:10; 2:1 +l to +b 1:10; 1.5:1 +l to +b 1:10; +l and <<<<
>>>>+b 1:1 +l to +b 1:10. +l The optimum ratios

0138 of primer to template may vary depending on the primer, mRNA, reverse

0139 transcription enzyme and reaction conditions (annealing temperature,

0140 buffering salts, etc.). The desired primer to template ratios can be <<<<
>>>>readily

0141 determined by one skilled in the art.

0142 +p In conventional methods of cDNA library construction, annealing or

0143 hybridizing primer to template is not carried out at a temperature which

0144 prevents, inhibits, reduces or substantially reduces internal priming.

0145 Typically, the mixture (e.g., mRNA and oligo(dT) primer) is chilled <<<<
>>>>on ice

0146 after denaturation or heating. This process typically causes annealing or

0147 hybridization of the primer to internal sites. According to a <<<<

>>>>preferred aspect of

0148 the present invention, the temperature during the annealing or <<<<

>>>>hybridization

0149 between the primer and the template is maintained so that internal <<<<

>>>>priming is

0150 inhibited, prevented, reduced or substantially reduced. In accordance <<<<

>>>>with the

0151 invention, such a result is accomplished by carrying out primer <<<<

>>>>annealing or

0152 hybridization at higher temperatures. Such conditions may also reduce the

0153 formation of mRNA secondary structures during cDNA synthesis. Preferably,

0154 temperatures for annealing or hybridizing primers to the templates <<<<

>>>>range from

0155 about +b 10+l +20 +0 C. to about +b 90+l +20 +0 C.; more preferably <<<<

>>>>about +b 10+l +20 +0 C. to about +b 80+l +20 +0 C.; still

0156 more preferably about +b 20+l +20 +0 C. to about +b 75+l +20 +0 C.; <<<<

>>>>more preferably about +b 25+l +20 +0 C. to

0157 about +b 75+l +20 +0 C.; still more preferably about +b 30+l +20 +0 <<<<

>>>>C. to about +b 65+l +20 +0 C.; still more

0158 preferably about +b 37+l +20 +0 C. to about +b 60+l +20 +0 C.; still <<<<

>>>>more preferably about +b 40+l +20 +0 C. to

0159 about +b 60+l +20 +0 C.; still more preferably about +b 45+l +20 +0 <<<<

>>>>C. to about +b 60+l +20 +0 C.; still more

0160 +pg,7

0161 preferably about +b 45+l +20 +0 C. to about +b 55+l +20 +0 C.; and <<<<
>>>>most preferably about +b 45+l +20 +0 C. to about

0162 +b 65+l +20 +0 C. The temperature used may vary depending on the type <<<<
>>>>and amount of

0163 primer and template and depending on the temperature optimum of the <<<<
>>>>reverse

0164 transcription enzyme. The optimum temperature or temperature ranges <<<<
>>>>can be

0165 readily determined by one skilled in the art.

0166 +p Conventional methods for cDNA synthesis typically requires the use of

0167 oligo(dT) primers of a particular length (+b 12+14 18 +l bases or <<<<
>>>>mer). Such primer

0168 length, however, lowers specificity of the primer thereby allowing <<<<
>>>>internal

0169 priming. Thus, the invention also relates to increasing specificity <<<<
>>>>of the

0170 primers to prevent, inhibit, reduce or substantially reduce internal <<<<
>>>>priming. In

0171 a preferred aspect, primer specificity is increased by increasing the <<<<
>>>>length of

0172 the primer. Thus, for cDNA synthesis, longer oligo(dT) primers may be <<<<
>>>>used

0173 in accordance with the invention. Preferably, primer length ranges <<<<
>>>>from about

0174 +b 20 +l to about +b 100 +l bases, about +b 20 +l to about +b 75 +l <<<<
>>>>bases, about +b 20 +l to about +b 60 +l bases,

0175 and about +b 20 +l to about +b 50 +l bases; more preferably about +b <<<<
>>>>20 +l to about +b 45 +l bases;

0176 more preferably about +b 20 +l to about +b 40 +l bases; and most <<<<
>>>>preferably about +b 25 +l to
0177 about +b 35 +l bases. In a preferred aspect, the length of the <<<<
>>>>primers are greater than
0178 +b 19 +l bases; more preferably greater than about +b 20 +l bases; <<<<
>>>>more preferably greater
0179 than about +b 25 +l bases; and still more preferably greater than <<<<
>>>>about +b 30 +l bases.
0180 Such primer lengths refer to the length of the primers which anneal or
0181 hybridize to the template Optimum length and content (nucleotide <<<<
>>>>sequence)
0182 of the primers may vary depending on the type of template, the desired
0183 reaction conditions, and the reverse transcription enzyme. In <<<<
>>>>accordance with
0184 the invention, additional sequences and/or modified nucleotides may be
0185 included in the primers of the invention. For example, additional <<<<
>>>>sequences
0186 (which do not necessarily anneal or hybridize to the template) may be <<<<
>>>>included
0187 in the primers of the invention to assist in cDNA synthesis including
0188 sequences comprising one or more restriction endonuclease sites, one <<<<
>>>>or more
0189 derivative nucleotides (e.g., hapten containing nucleotides such as <<<<
>>>>biotinylated
0190 nucleotides), and the like. The type and length of the primers used in

0191 +pg,8

0192 accordance with the invention can be readily determined by one or more

0193 skilled in the art.

0194 +p Conventional cDNA synthesis methods do not control or vary activity

0195 of the reverse transcription enzyme to optimize the reverse transcription

0196 reaction. In accordance with the invention, the activity of the reverse

0197 transcriptase is preferably controlled to start synthesis at a <<<<

>>>>desired time in the

0198 reaction. In a preferred aspect, reverse transcriptase activity is <<<<

>>>>inhibited or

0199 prevented until optimum or desired reaction conditions are achieved. <<<<

>>>>Such a

0200 result is accomplished in accordance with the invention by the use of

0201 inhibitors (such as antibodies or antibody fragments) which inhibit <<<<

>>>>reverse

0202 transcriptase activity. Such reverse transcriptase inhibitors prevent <<<<

>>>>or inhibit

0203 reverse transcriptase activity at low temperatures such that internal <<<<

>>>>priming is

0204 prevented, inhibited, reduced or substantially reduced. In accordance <<<<

>>>>with the

0205 invention, such inhibitors preferably prevent reverse transcriptase <<<<

>>>>activity

0206 below +b 35+l +20 +0 C., below +b 40+l +20 +0 C., below +b 45+l +20 <<<<

>>>>+0 C., below +b 50+l +20 +0 C., below +b 55+l +20 +0 C., below +b 60+l <<<<

>>>>+20 +0 C.,

0207 below +b 65+l +20 +0 C., below +b 70+l +20 +0 C., below +b 75+l +20 <<<<

>>>>+0 C., below +b 80+l +20 +0 C., below +b 85+l +20 +0 C. and below

0208 +b 90+l +20 +0 C. Depending on the thermostability of the enzyme <<<<

>>>>having reverse

0209 transcriptase activity, the inhibitor may be designed to inhibit <<<<

>>>>activity of the

0210 enzyme at a point at or near the temperature optimum for the enzyme of

0211 interest. Preferably, the inhibitor is inactivated at a temperature <<<<

>>>>below or near

0212 the temperature optimum of the enzyme used, thereby allowing reverse

0213 transcription to take place. Thus, the invention generally relates to <<<<

>>>>the use of

0214 reverse transcriptase inhibitors in cDNA synthesis. The type and <<<<

>>>>amount of

0215 inhibitor may vary depending on the type and amount of reverse <<<<

>>>>transcription

0216 enzyme and depending on the reaction conditions to be used. The type of

0217 inhibitor and conditions used with such inhibitor can be readily <<<<

>>>>determined by

0218 one of ordinary skill in the art.

0219 +p In accordance with the invention, any one or a combination of the

0220 above improvements to cDNA synthesis may be used. Using any one or a

0221 combination of these improvements provides for improved first strand cDNA

+pg,9

synthesis (e.g., more total cDNA, larger cDNA and/or more full-length cDNA). In accordance with the invention, the first strand cDNA molecules may be used as templates to make one or more double stranded nucleic acid molecules (e.g., double strand cDNA molecules) by incubating one or <<<<>>>>more of the first strand cDNA molecules produced by the methods of the invention under conditions sufficient to make one or more nucleic acid molecules complementary to all or a portion of the first strand cDNA molecules.

Conditions for making double stranded nucleic acid molecules preferably include incubation with one or more components consisting of one or more DNA polymerases, one or more nucleotides, one or more buffering salts,<<<<>>>> and one or more primers. In another aspect of the invention, such <<<<>>>>conditions are modified to provide an increase in the total amount of double <<<<>>>>stranded cDNA produced, an increase in the length or size of the double stranded cDNA molecule produced, and/or an increase in percentage full-length double stranded cDNA molecule produced. Preferably, such conditions relate to optimization of ribonuclease (RNase) digestion after first strand cDNA synthesis. During first strand cDNA synthesis, if a full-length cDNA <<<<>>>>molecule complementary to the mRNA template is not made, a single stranded mRNA containing the cap structure will be present at the +b 5+1 +40 +0 end <<<<>>>>of the mRNA of the mRNA/cDNA hybrid. If a full-length cDNA is produced, a double stranded mRNA/cDNA hybrid is produced with no single stranded mRNA present. Preferably, such digestion conditions are optimized so that the <<<<

>>>>single stranded

0245 mRNA of the mRNA/cDNA double stranded molecules formed during first

0246 strand cDNA synthesis is subject to RNase digestion. In this manner, cap

0247 structure from mRNA/cDNA hybrids which are not full-length are removed

0248 while full-length mRNA/cDNA hybrids will retain the cap structure. Thus,

0249 cap capture can be used to select for full-length molecules and <<<<

>>>>select against

0250 molecules which are not full-length. In a preferred aspect, the <<<<

>>>>conditions are

0251 such that the single stranded mRNA of the mRNA/cDNA hybrid is digested or

0252 degraded while the mRNA of the double stranded mRNA/cDNA hybrid is not

0253 +pg,10

0254 degraded or not substantially degraded. Thus, such RNase digestion is

0255 conducted under conditions such that second strand synthesis is not

0256 substantially adversely affected. That is, second strand synthesis in

0257 accordance with the invention produces larger double stranded cDNA

0258 molecules compared to conventional techniques. Conventional RNase I

0259 conditions typically range from +b 25 +l u/+82 g to +b 40 +l u/+82 g <<<<

>>>>mRNA at +b 37+l +20 +0 C. and RNase

0260 A conditions typically are +b 1000 +l ng/+82 g mRNA at +b 37+l +20 +0 <<<<

>>>>C. Using conventional

0261 RNase digestion, the average size of double stranded cDNA molecules

0262 produced is about +b 200 +l bases. According to the present invention <<<<

>>>>the average

0263 size of double stranded cDNA molecules produced is preferably greater <<<<

>>>>than

0264 about +b 300 +l bases, greater than about +b 400 +l bases, greater <<<<

>>>>than about +b 500 +l bases,

0265 greater than about +b 600 +l bases, greater than about +b 700 +l <<<<

>>>>bases, greater than about

0266 +b 800 +l bases, greater than about +b 900 +l bases, greater than <<<<

>>>>about +b 1 +l kilobase, greater

0267 than about +b 1.5 +l kilobases, and greater than about +b 2 +l <<<<

>>>>kilobases. In one

0268 embodiment of the invention, the concentration of the ribonuclease, <<<<

>>>>the type of

0269 ribonuclease and reaction conditions are optimized to improve double <<<<

>>>>stranded

0270 cDNA synthesis in accordance with the invention. Preferred ribonuclea<<<<

>>>>ses for

0271 use in ribonuclease digestions include ribonuclease A (RNase A) and/or

0272 ribonuclease I (RNase I). Generally, lower temperatures (about +b 4+l <<<<

>>>>+20 +0 C. to about

0273 +b 50+l +20 +0 C.) and higher salt concentrations (about +b 5 +l mM <<<<

>>>>to about +b 5 +l M) will assist in

0274 inhibiting or controlling RNase digestion in accordance with the <<<<

>>>>invention.

0275 Salts used may include sodium chloride, potassium, chloride, magnesium

0276 chloride, sodium acetate etc. Additionally, lowering RNase amounts or

0277 concentrations may be used to accomplish the desired result. Such

0278 concentrations for RNase A may range from about +b 0.001 +l ng/+82 g <<<<

>>>>mRNA to

0279 about +b 500 +l ng/+82 g of mRNA and for RNase I may range from about <<<<

>>>>+b 0.001 +l u/+82 g

0280 mRNA to about +b 500 +l u/+82 g mRNA. The incubation temperature, RNase

0281 concentration and salt concentration may be readily determined by one <<<<

>>>>skilled

0282 in the art. In a preferred aspect, concentration of the RNase A <<<<

>>>>include ranges

0283 from +b 0.1 +l ng/+82 g mRNA to +b 10 +l ng/+82 g mRNA in TE buffer <<<<

>>>>(+b 10 +l mM Tris, pH +b 7.5,

0284 +pg,11

0285 1 +l mM EDTA) at +b 37+l +20 +0 C. Alternatively, the concentration <<<<
>>>>of the RNase A can

0286 include ranges from +b 0.1 +l ng/+82 g mRNA to +b 500 +l ng/+82 g <<<<
>>>>mRNA in +b 10 +l mM Tris, pH

0287 +b 7.5 +l buffer containing +b 250 +l mM NaCl at +b 25+l +20 +0 C. <<<<
>>>>for +b 30 +l minutes. Preferably,

0288 concentration of the RNase I used ranges from +b 0.1 +l unit/+82 g <<<<
>>>>mRNA to +b 1.0 +l

0289 unit/+82 g mRNA in +b 10 +l mM Tris-HCl (pH +b 7.5+l), +b 5 +l mM <<<<
>>>>EDTA (pH +b 8.0+l), +b 200 +l mM

0290 sodium acetate at +b 37+l +20 +0 C. Alternatively, the concentration <<<<
>>>>of the RNase I can be

0291 used at ranges from +b 1.0 +l unit/+82 g mRNA to +b 100 +l units/+82 <<<<
>>>>g mRNA in the same

0292 buffer at +b 25+l +20 +0 C. for +b 30 +l minutes.

0293 +p In another aspect, the invention relates to capture or binding of <<<<
>>>>the cap

0294 structure (e.g., m+hu 7+l GpppN) of the mRNA before, during or after <<<<
>>>>first strand

0295 cDNA synthesis. Thus, the invention relates to selection of mRNA (before

0296 first strand synthesis) or mRNA/cDNA hybrids (after or during first <<<<
>>>>strand

0297 synthesis) which have the cap structure in carrying out the methods <<<<
>>>>of the

0298 invention. Such selection or capture may be accomplished with any cap

0299 binding molecule such as eIF+b 4+l E, eIF+b 4+l E peptides, eIF+b 4+l <<<<
>>>>E peptide fragments (see

0300 WO +b 98/08865+l) and antibodies or antibody fragments specific for <<<<

>>>>cap structure.

0301 In a preferred aspect, selection of the cap structure is accomplished <<<<
>>>>after first

0302 strand synthesis. More preferably, such cap capture occurs after <<<<
>>>>ribonuclease

0303 digestion in accordance with the methods of the invention. For example,

0304 mRNA/cDNA hybrids subjected to ribonuclease digestion are captured and

0305 then used for second strand cDNA synthesis according to the invention.

0306 +p Thus, the present invention is generally directed to methods of

0307 synthesizing nucleic acid molecules. The present invention is more

0308 specifically directed to methods of making one or more nucleic acid

0309 molecules, especially cDNA molecules or cDNA libraries, comprising mixing

0310 one or more nucleic acid templates (preferably mRNA, poly A RNA or a

0311 population of mRNA molecules) with at least one polypeptide having <<<<
>>>>reverse

0312 transcriptase activity, and incubating the mixture under conditions <<<<
>>>>sufficient to

0313 make one or more first nucleic acid molecules (e.g., first strand cDNA)

0314 complementary to all or a portion of the one or more nucleic acid <<<<
>>>>templates.

0315 +pg,12

0316 +p In accordance with the invention, such conditions provide for an <<<<
>>>>increased

0317 total amount of nucleic acid molecule (cDNA) produced, compared to

0318 conventional procedures which do not employ the improved modifications or

0319 conditions of the invention. The invention also provides for an <<<<
>>>>increase of

0320 length or average size of the nucleic acid molecules (cDNA) produced <<<<
>>>>and/or

0321 an increase in the percentage or amount of full-length nucleic acid <<<<
>>>>molecules

0322 (cDNA) produced, compared to conventional procedures which do not employ

0323 the improved modifications or conditions of the invention. Determinin<<<<
>>>>g the

0324 amount, length and full-length content of the cDNA produced can be

0325 determined by conventional techniques well known in the art and as <<<<
>>>>described

0326 herein. The percentage or average percentages of full-length cDNA in cDNA

0327 libraries produced in accordance with the invention are preferably <<<<
>>>>above about

0328 +b 15+l %, more preferably above about +b 20+l %, more preferably <<<<
>>>>above about +b 25+l %,

0329 more preferably above about +b 30+l %, more preferably above about +b <<<<
>>>>40+l %, more

0330 preferably above about +b 50+l %, more preferably above about +b 60+l <<<<
>>>>%, more

0331 preferably above about +b 70+l %, more preferably above about +b 80+l <<<<
>>>>% and most

0332 preferably above about +b 90+l % Such full-length percentages are <<<<

>>>>preferably

0333 determined by random selection of a portion of the clones of the cDNA <<<<

>>>>library

0334 of interest (e.g., +b 100 +l to +b 1000 +l clones), sequencing the <<<<

>>>>clones and comparing the

0335 sequences to known sequence data bases.

0336 +p In preferred aspects of the invention, the improved results of the

0337 invention are preferably accomplished by one or a combination of

0338 modifications to the conditions for nucleic acid or cDNA synthesis. Such

0339 conditions preferably include modifications for improving first <<<<

>>>>strand cDNA

0340 synthesis and/or improving second strand cDNA synthesis.

0341 +p In a preferred aspect, the invention specifically relates to <<<<

>>>>methods of

0342 making one or more double stranded cDNA molecules comprising incubating

0343 one or more mRNA molecules preferably a population of mRNA molecules)

0344 with one or more primers of the invention at temperatures and primer

0345 concentrations to prevent, inhibit, reduce or substantially reduce <<<<

>>>>internal

0346 +pg,13

0347 priming prior to or during first strand cDNA synthesis. Such reaction is

0348 preferably conducted in the presence of one or more inhibitors of reverse

0349 transcriptase activity in accordance with the invention. Ribonuclease <<<<

>>>>digestion

0350 is preferably conducted before second strand cDNA synthesis and at

0351 ribonuclease concentrations sufficient to increase the length, amount <<<<

>>>>and/or

0352 size of double stranded cDNA molecules produced during second strand

0353 synthesis. In accordance with the invention, cap capture is preferably

0354 accomplished during or after the ribonuclease digestion.

0355 +p The invention is also directed to nucleic acid molecules and cDNA

0356 molecules or populations of cDNA molecules (single or double-stranded)

0357 produced according to the above-described methods and to vectors

0358 (particularly expression vectors) comprising these nucleic acid <<<<

>>>>molecules and

0359 cDNA molecules. The invention also relates to host cells containing such

0360 cDNA molecules and/or vectors.

0361 +p The invention is also directed to kits for use in the methods of the

0362 invention. Such kits can be used for making single or double-stranded <<<<

>>>>nucleic

0363 acid molecules. The kits of the invention comprise a carrier, such as <<<<

>>>>a box or

0364 carton, having therein one or more containers, such as vials, tubes, <<<<

>>>>bottles and

0365 the like. Such kits may comprise at least one component selected from the

0366 group consisting of primers (preferably primers having higher <<<<

>>>>specificity and

0367 most preferably oligo(dT) primers having a length equal to or greater <<<<

>>>>than +b 20 +l

0368 bases), one or more polypeptides having reverse transcriptase <<<<

>>>>activity (reverse

0369 transcriptases and DNA polymerases), one or more inhibitors of reverse

0370 transcription (e.g., antibodies and antibody fragments directed against

0371 polypeptides having RT activity), one or more cap binding molecules <<<<

>>>>(e.g.,

0372 antibodies or antibody fragments directed against cap structure), <<<<

>>>>nucleic acid

0373 synthesis reaction buffers, one or more nucleotides, one or more <<<<

>>>>vectors, and

0374 instructions for carrying out the methods of the invention.

0375 +p The invention also relates to compositions for use in the invention or

0376 made while carrying out the methods of the invention. Such compositions

0377 +pg,14

0378 may comprise at least one primer (e.g., oligo(dT) or derivatives <<<<
>>>>thereof) and at

0379 least one template in a sample or reaction mixture in amounts or <<<<
>>>>ratios in

0380 accordance with the invention. Such composition may further comprise <<<<
>>>>one or

0381 more polypeptides having reverse transcriptase activity, one or more <<<<
>>>>reverse

0382 transcription inhibitors (e.g., anti-RT antibodies or fragments <<<<
>>>>thereof), one or

0383 more nucleotides, one or more cap binding molecules (e.g., anti-cap <<<<
>>>>antibodies

0384 for fragments thereof), one or more buffering salts and the like. Such

0385 compositions may also be maintained at a temperature to avoid internal

0386 priming in accordance with the invention.

0387 +p The compositions of the invention may also comprise amounts of

0388 ribonuclease in accordance with the invention. Such compositions may <<<<
>>>>further

0389 comprise at least one component selected from one or more mRNA/cDNA

0390 hybrids, one or more nucleotides, one or more polypeptides having reverse

0391 transcriptase activity, one or more buffering salts, one or more cap <<<<
>>>>binding

0392 molecules (e.g., anti-cap antibodies or fragments thereof) and the like.

0393 +p The invention also relates to one or more antibodies (monoclonal and

0394 polyclonal) and fragments thereof for use in the methods, composition<<<<
>>>>s and

0395 kits of the invention. Such antibodies, include anti-cap and/or anti-RT

0396 antibodies and antibody fragments

0397 Other preferred embodiments of the present invention will be apparent
0398 to one of ordinary skill in the art in view of the following drawings and
0399 description of the invention.

0400 +dr +cl BRIEF DESCRIPTION OF THE DRAWINGS

0401 +p FIG. 1 is an autoradiograph of first strand cDNA synthesized with
0402 SuperScript+198 +0 II (SS II) RT at +b 45+l +20 +0 C. with a +e,fra <<<<
>>>>+b 5/6+l +ee +0 Kb template with molar ratios
0403 of oligo(dT)+hd 25-30+l /mRNA of +b 1:1, 2.5:1, 5:1, 10:1, +l and +b <<<<
>>>>50:1.

0404 +pg,15

0405 +p FIG. 2 is an autoradiograph of first strand cDNA synthesized with

0406 ThermoScript+198 +0 II (TS II) RT at +b 45+l +20 +0 C., +b 50+l +20 <<<<

>>>>+0 C. and +b 55+l +20 +0 C. with a +e,fra +b 5/6+l +ee +0 Kb template

0407 with molar ratios of oligo (dT)+hd 25-30+l /mRNA of +b 1:1, 2.5:1, <<<<

>>>>5:1, 10:1, +l and +b 50:1.

0408 +p FIG. 3 is an autoradiograph of first strand cDNA synthesized with SS

0409 II RT using standard reaction temperatures and varying reaction <<<<

>>>>temperatures

0410 with a molar ratio of biotinylated-Not I-oligo(dT)+hd 25+l /mRNA of <<<<

>>>>+b 0:1, 1:1 +l and

0411 +b 15:1.

0412 +p FIG. 4 is an autoradiograph of first strand cDNA synthesized with TS

0413 II RT using standard reaction conditions in which the primer/template

0414 annealing is incubated on ice prior to cDNA synthesis and using <<<<

>>>>conditions

0415 according to the invention in which annealing and the synthesis reaction

0416 temperatures are maintained above +b 30+l +20 +0 C. (preferably above <<<<

>>>>+b 37+l +20 +0 C.) with a molar

0417 ratio of biotinylated-Not I-oligo(dT)+hd 25+l /mRNA of +b 1:1 +l and <<<<

>>>>+b 15:1. +l Maintaining

0418 the annealing and reaction temperatures above +b 30+l +20 +0 C. <<<<

>>>>(preferably above +b 37+l +20 +0 C.)

0419 in accordance with the invention may also be referred to as +37 hot <<<<

>>>>start.+38

0420 +p FIG. 5 is an autoradiograph of second strand cDNA synthesized

0421 using different amounts of RNase A.

0422 +p FIG. 6 is an autoradiograph of second strand cDNA synthesized

0423 using different amounts of RNase I.

0424 +de +cl DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

0425 +ps Definitions:

0426 +p In order to provide a clearer and consistent understanding of the

0427 specification and claims, including the scope to be given such terms, the

0428 following definitions are provided.

0429 +p Internal priming as used herein refers to hybridization or <<<<

>>>>annealing of

0430 one or more primers at one or more sites within one or more mRNA <<<<

>>>>molecules

0431 other than at the poly A tail located at the +b 3+l +40 +0 termini of <<<<

>>>>the mRNA molecule.

0432 +pg,16

0433 +p Library as used herein refers to a set of nucleic acid molecules

0434 (circular or linear) which is representative of all or a portion or <<<<

>>>>significant

0435 portion of the DNA content of an organism (a +37 genomic library+38),<<<<

>>>> or a set of

0436 nucleic acid molecules representative of all or a portion or <<<<

>>>>significant portion

0437 of the expressed genes (a +37 cDNA library+38) in a cell, tissue, <<<<

>>>>organ or organism.

0438 Such libraries may or may not be contained in one or more vectors.

0439 +p Vector as used herein refers to a plasmid, cosmid, phagemid or phage

0440 DNA or other DNA molecule which is able to replicate autonomously in a

0441 host cell, and which is characterized by one or a small number of <<<<

>>>>restriction

0442 endonuclease recognition sites at which such DNA sequences may be cut <<<<

>>>>in a

0443 determinable fashion without loss of an essential biological function <<<<

>>>>of the

0444 vector, and into which DNA may be inserted in order to bring about its

0445 replication and cloning. The vector may further contain one or more <<<<

>>>>markers

0446 suitable for use in the identification of cells transformed with the <<<<

>>>>vector.

0447 Markers, for example, include but are not limited to tetracycline <<<<

>>>>resistance or

0448 ampicillin resistance. Such vectors may also contain one or more

0449 recombination sites, one or more termination sites, one or more <<<<

>>>>origins of

0450 replication, and the like.

0451 +p Primer as used herein refers to a single-stranded oligonucleotide that

0452 is extended by covalent bonding of nucleotide monomers during <<<<

>>>>amplification

0453 or polymerization of a DNA molecule. Preferred primers for use in the

0454 invention include oligo(dT) primers or derivatives or variants thereof.

0455 +p Oligonucleotide as used herein refers to a synthetic or natural

0456 molecule comprising a covalently linked sequence of nucleotides which are

0457 joined by a phosphodiester bond between the +b 3+1 +40 +0 position of <<<<

>>>>the deoxyribose or

0458 ribose of one nucleotide and the +b 5+1 +40 +0 position of the <<<<

>>>>deoxyribose or ribose of the

0459 adjacent nucleotide.

0460 +p Template as used herein refers to double-stranded or single-stranded

0461 nucleic acid molecules which are to be amplified, synthesized or <<<<

>>>>sequenced.

0462 In the case of a double-stranded molecules, denaturation of its <<<<

>>>>strands to form

+pg,17
a first and a second strand is preferably performed before these <<<<
>>>>molecules may
be amplified, synthesized or sequenced, or the double stranded <<<<
>>>>molecule may
be used directly as a template. For single stranded templates, a primer,
complementary to a portion of the template is hybridized or annealed <<<<
>>>>under
appropriate conditions and one or more polymerases or reverse <<<<
>>>>transcriptases
may then synthesize a nucleic acid molecule complementary to all or a <<<<
>>>>portion
of said template. The newly synthesized molecules, according to the <<<<
>>>>invention,
may be equal or shorter in length than the original template.
+p Incorporating as used herein means becoming a part of a DNA and/or
RNA molecule or primer.
+p Amplification as used herein refers to any in vitro method for
increasing the number of copies of a nucleotide sequence with the use <<<<
>>>>of a
polymerase. Nucleic acid amplification results in the incorporation of
nucleotides into a DNA and/or RNA molecule or primer thereby forming a
new molecule complementary to a template. The formed nucleic acid
molecule and its template can be used as templates to synthesize <<<<
>>>>additional
nucleic acid molecules. As used herein, one amplification reaction may
consist of many rounds of replication. DNA amplification reactions <<<<
>>>>include,
for example, polymerase chain reactions (PCR). One PCR reaction may

0483 consist of +b 5 +l to +b 100 +l +37 cycles+38 +0 of denaturation and <<<<
>>>>synthesis of a DNA molecule.

0484 +p Nucleotide as used herein refers to a base-sugar-phosphate

0485 combination. Nucleotides are monomeric units of a nucleic acid sequence

0486 (DNA and RNA). The term nucleotide includes ribonucleoside triphosphate

0487 ATP, UTP, CTG, GTP and deoxyribonucleoside triphosphates such as dATP,

0488 dCTP, dITP, dUTP, dGTP, dTTP, or derivatives thereof. Such derivatives

0489 include, for example, +8 +60 S+9 dATP, +b 7+l -deaza-dGTP, +b 7+l -<<<<
>>>>deaza-dATP, and

0490 biotinylated or haptenylated nucleotides. The term nucleotide as used <<<<
>>>>herein

0491 also refers to dideoxyribonucleoside triphosphates (ddNTPs) and their

0492 derivatives. Illustrated examples of dideoxyribonucleoside triphosphates

0493 include, but are not limited to, ddATP, ddCTP, ddGTP, ddITP, and ddTTP.

0494 +pg,18

0495 According to the present invention, a +37 nucleotide+38 +0 may be <<<<
>>>>unlabeled or
0496 detectably labeled by well known techniques. Detectable labels <<<<
>>>>include, for
0497 example, radioactive isotopes, fluorescent labels, chemiluminescent <<<<
>>>>labels,
0498 bioluminescent labels and enzyme labels.

0499 +p Hybridization or annealing as used herein refers to base pairing of
0500 two complementary single-stranded nucleic acid molecules (RNA and/or
0501 DNA) to give a double-stranded molecule. As used herein, two nucleic acid
0502 molecules may be hybridized or annealed, although the base pairing is not
0503 completely complementary. Accordingly, mismatched bases do not prevent
0504 hybridization or annealing of two nucleic acid molecules provided that
0505 appropriate conditions, well known in the art, are used. In the present
0506 invention, the term hybridization or annealing preferably refers to
0507 hybridization of one or more primers (e.g., oligo(dT) or derivatives <<<<
>>>>thereof) to
0508 one or more templates (e.g., mRNA).

0509 +p Host cell as used herein refers to any prokaryotic or eukaryotic cell
0510 that is the recipient of a replicable expression vector or cloning <<<<
>>>>vector. The
0511 terms +37 host+38 +0 or +37 host cell+38 +0 may be used interchangeab<<<<
>>>>ly herein. For examples
0512 of such hosts, see Maniatis et al., +37 Molecular Cloning: A Laboratory
0513 Manual,+38 +0 Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
0514 (+b 1982+l). Preferred prokaryotic hosts include, but are not <<<<
>>>>limited to, bacteria of
0515 the genus Escherichia (e.g., +i E. coli+l), Bacillus, Staphylococcus,<<<<

>>>> Agrobacter

0516 (e.g., +i A. tumefaciens+l), Streptomyces, Pseudomonas, Salmonella, <<<<

>>>>Serratia,

0517 Caryophanon, etc. The most preferred prokaryotic host is +i E. coli. <<<<

>>>>+l Bacterial

0518 hosts of particular interest in the present invention include +i E. <<<<

>>>>coli +l strains K+b 12, +l

0519 DH+b 10+l B, DH+b 5+l +60 , Stb+b 12 +l and HB+b 101, +l and others <<<<

>>>>available from Life

0520 Technologies, Inc. Preferred eukaryotic hosts include, but are not <<<<

>>>>limited to,

0521 fungi, fish cells, yeast cells, plant cells and animal cells. <<<<

>>>>Particularly preferred

0522 animal cells are insect cells such as Drosophila cells, Spodoptera <<<<

>>>>Sf+b 9, +l Sf+b 21 +l

0523 cells and Trichoplusa High-Five cells; nematode cells such as +i C. <<<<

>>>>elegans +l cells;

0524 +pg,19

0525 and mammalian cells such as COS cells, CHO cells, VERO cells, +b 293 <<<<
>>>>+l cells,

0526 PERC+b 6 +l cells, BHK cells and human cells.

0527 +p Expression vector as used herein refers to a vector which is <<<<
>>>>capable of

0528 enhancing the expression of a gene or portion of a gene which has <<<<
>>>>been cloned

0529 into it, after transformation or transfection into a host cell. The <<<<
>>>>cloned gene is

0530 usually placed under the control (i.e., operably linked to) certain <<<<
>>>>control

0531 sequences such as promoter sequences. Such promoters include but are not

0532 limited to phage lambda P+hd L +l promoter, and the +i E. coli +l lac,<<<<
>>>> trp and tac

0533 promoters. Other suitable promoters will be known to the skilled artisan.

0534 +p The nucleic acid templates suitable for reverse transcription <<<<
>>>>according

0535 to the invention include any nucleic acid molecule or populations of <<<<
>>>>nucleic

0536 acid molecules (preferably one or more RNA molecules (e.g., one or more

0537 mRNA molecules or poly A+hu +30 +l +0 RNA molecules, and more <<<<
>>>>preferably a

0538 population of mRNA molecules) or one or more DNA molecules), particularly

0539 those derived from a cell or tissue. In a preferred aspect, a <<<<
>>>>population of

0540 mRNA molecules (a number of different mRNA molecules) are used to make

0541 a cDNA library according to the present invention.

0542 +p To make the nucleic acid molecule or molecules complementary to the

0543 one or more templates, a primer (e.g., an oligo(dT) primer) and one <<<<
>>>>or more
0544 nucleotides are used for nucleic acid synthesis typically in the +b <<<<
>>>>3+l +40 +0 to +b 5+l +40 +0
0545 direction. Nucleic acid molecules suitable for reverse transcription <<<<
>>>>according
0546 to this aspect of the invention include any nucleic acid molecule, <<<<
>>>>particularly
0547 those derived from a prokaryotic or eukaryotic cell. Such cells may <<<<
>>>>include
0548 normal cells, diseased cells, transformed cells, established cells, <<<<
>>>>progenitor
0549 cells, precursor cells, fetal cells, embryonic cells, bacterial cells,<<<<
>>>> yeast cells,
0550 animal cells (including human cells), avian cells, plant cells and <<<<
>>>>the like, or
0551 tissue isolated from a plant (e.g., corn, tomato, tobacco, potato, <<<<
>>>>soy bean, etc.)
0552 or an animal (e.g., human, cow, pig, mouse, sheep, horse, monkey, canine,
0553 feline, rat, rabbit, bird, fish, insect, etc.). Such nucleic acid <<<<
>>>>molecules may
0554 also be isolated from viruses.

+pg,20

+p The nucleic acid molecules that are used as templates to prepare cDNA molecules according to the methods of the present invention are <<<<>>>>preferably obtained from natural sources, such as a variety of cells, tissues, <<<<>>>>organs or organisms. Cells that may be used as sources of nucleic acid <<<<>>>>molecules may be prokaryotic (bacterial cells, including but not limited to those <<<<>>>>of species of the genera Escherichia, Bacillus, Serratia, Salmonella, Staphylococcus, Streptococcus, Clostridium, Chlamydia, Neisseria, Treponema, Mycoplasma, Borrelia, Legionella, Pseudomonas, Mycobacterium, Helicobacter, Erwinia, Agrobacterium, Rhizobium, Xanthomonas and Streptomyces) or eukaryotic (including fungi (especially yeasts), plants, protozoans and other <<<<>>>>parasites, and animals including insects (particularly Drosophila spp. cells), nematodes (particularly +i Caenorhabditis elegans +1 cells), and mammals <<<<>>>>(particularly human cells)).

+p Mammalian somatic cells that may be used as sources of nucleic acids include blood cells (reticulocytes and leukocytes), endothelial cells,<<<<>>>> epithelial cells, neuronal cells (from the central or peripheral nervous <<<<>>>>systems), muscle cells (including myocytes and myoblasts from skeletal, smooth or cardiac muscle), connective tissue cells (including fibroblasts, adipocytes, chondrocytes, chondroblasts, osteocytes and osteoblasts) and other <<<<>>>>stromal

cells (e.g., macrophages, dendritic cells, Schwann cells). Mammalian germ cells (spermatocytes and oocytes) may also be used as sources of <<<<>>>>nucleic acids for use in the invention, as may the progenitors, precursors and stem <<<<>>>>cells that give rise to the above somatic and germ cells. Also suitable for use <<<<>>>>as nucleic acid sources are mammalian tissues or organs such as those derived from brain, kidney, liver, pancreas, blood, bone marrow, muscle, nervous, <<<<>>>>skin, genitourinary, circulatory, lymphoid, gastrointestinal and connective <<<<>>>>tissue sources, as well as those derived from a mammalian (including human) embryo or fetus.

+p Any of the above cells, tissues and organs may be normal, diseased, transformed, established, progenitors, precursors, fetal or embryonic.

0586 +pg,21

0587 Diseased cells may, for example, include those involved in infectious <<<<
>>>>diseases

0588 (caused by bacteria, fungi or yeast, viruses (including AIDS, HIV, HTLV,

0589 herpes, hepatitis and the like) or parasites), in genetic or biochemical

0590 pathologies (e.g., cystic fibrosis, hemophilia, Alzheimer+3 s disease,<<<<
>>>> muscular

0591 dystrophy or multiple sclerosis) or in cancerous processes. <<<<
>>>>Transformed or

0592 established animal cell lines may include, for example, COS cells, <<<<
>>>>CHO cells,

0593 VERO cells, BHK cells, HeLa cells, HepG+b 2 +l cells, K+b 562 +l <<<<
>>>>cells, +b 293 +l cells, L+b 929 +l

0594 cells, F+b 9 +l cells, and the like. Other cells, cell lines, tissues,<<<<
>>>> organs and

0595 organisms suitable as sources of nucleic acids for use in the present <<<<
>>>>invention

0596 will be apparent to one of ordinary skill in the art.

0597 +p Once the starting cells, tissues, organs or other samples are <<<<
>>>>obtained,

0598 nucleic acid molecules (such as mRNA) may be isolated therefrom by

0599 methods that are well-known in the art (See, e.g., Maniatis, T., et <<<<
>>>>al., +i Cell

0600 +b 15:687+14 701 +l (+b 1978+l); Okayama, H., and Berg, P., +i Mol. <<<<
>>>>Cell. Biol. +b 2:161+14 170 +l

0601 (+b 1982+l); Gubler, U., and Hoffman, B. J., +i Gene +b 25:263+14 <<<<
>>>>269 +l (+b 1983+l); and Message

0602 Maker+198 +0 mRNA Isolation System available from Life Technologies, <<<<
>>>>Inc.).

0603 The nucleic acid molecules thus isolated may then be used to prepare cDNA

0604 molecules and cDNA libraries in accordance with the present <<<<

>>>>invention. The

0605 cDNA molecules and/or cDNA libraries produced in accordance with the

0606 invention are preferably contained in one or more vectors. Such <<<<

>>>>vectors may

0607 be introduced into one or more host cells by standard transformation or

0608 transfection techniques well known in the art. Preferred host cells <<<<

>>>>include

0609 prokaryotic host cells such as cells of the genus Escherichia, <<<<

>>>>particularly +i E.

0610 coli.

0611 +p Enzymes for use in the compositions, methods and kits of the invention

0612 include any enzyme having reverse transcriptase activity. Such enzymes

0613 include, but are not limited to, retroviral reverse transcriptase, <<<<

>>>>retrotransposon

0614 reverse transcriptase, hepatitis B reverse transcriptase, cauliflower <<<<

>>>>mosaic

0615 virus reverse transcriptase, bacterial reverse transcriptase, Tth DNA

0616 polymerase, Taq DNA polymerase (Saiki, R. K., et al., +i Science +b <<<<

>>>>239:487+14 491

0617 +pg,22

0618 +l (+b 1988+l); U.S. Pat. Nos. +b 4,889,818 +l and +b 4,965,188+l), <<<<
>>>>Tne DNA polymerase

0619 (WO +b 96/10640+l), Tma DNA polymerase (U.S. Pat. No. +b 5,374,553+l <<<<
>>>>) and

0620 mutants, fragments, variants or derivatives thereof (see, e.g., commonly

0621 owned, co-pending U.S. patent application Ser. No. +b 08/706,702 +l <<<<
>>>>and Ser. No. +b 08/706,706, +l

0622 both filed Sep. +b 9, 1996, +l which are incorporated by reference <<<<
>>>>herein in

0623 their entireties). As will be understood by one of ordinary skill in <<<<
>>>>the art,

0624 modified reverse transcriptases and DNA polymerase having RT activity may

0625 be obtained by recombinant or genetic engineering techniques that are <<<<
>>>>well-known

0626 in the art. Mutant reverse transcriptases or polymerases can, for

0627 example, be obtained by mutating the gene or genes encoding the reverse

0628 transcriptase or polymerase of interest by site-directed or random <<<<
>>>>mutagenesis.

0629 Such mutations may include point mutations, deletion mutations and

0630 insertional mutations. Preferably, one or more point mutations (e.g.,

0631 substitution of one or more amino acids with one or more different amino

0632 acids) are used to construct mutant reverse transcriptases or <<<<
>>>>polymerases for

0633 use in the invention. Fragments of reverse transcriptases or <<<<
>>>>polymerases may

0634 also be obtained by deletion mutation by recombinant techniques that <<<<
>>>>are well-known

0635 in the art, or by enzymatic digestion of the reverse transcriptase(s) or

0636 polymerase(s) of interest using any of a number of well-known proteolytic

0637 enzymes.

0638 +p Preferred enzymes for use in the invention include those that are

0639 reduced or substantially reduced in RNase H activity. Such enzymes <<<<

>>>>that are

0640 reduced or substantially reduced in RNase H activity may be obtained by

0641 mutating the RNase H domain within the reverse transcriptase of interest,

0642 preferably by one or more point mutations, one or more deletion <<<<

>>>>mutations,

0643 and/or one or more insertion mutations as described above. By an enzyme

0644 +37 substantially reduced in RNase H activity+38 +0 is meant that the <<<<

>>>>enzyme has less

0645 than about +b 30+l %, less than about +b 25+l %, less than about +b <<<<

>>>>20+l %, more preferably

0646 less than about +b 15+l %, less than about +b 10+l %, less than about <<<<

>>>>+b 7.5+l %, or less than

0647 about +b 5+l %, and most preferably less than about +b 5+l % or less <<<<

>>>>than about +b 2+l %, of

0648 +pg,23

0649 the RNase H activity of the corresponding wildtype or RNase H+hu +30 <<<<
>>>>+l +0 enzyme such

0650 as wildtype Moloney Murine Leukemia Virus (M-MLV), Avian

0651 Myeloblastosis Virus (AMV) or Rous Sarcoma Virus (RSV) reverse

0652 transcriptases. The RNase H activity of any enzyme may be determined by a

0653 variety of assays, such as those described, for example, in U.S. Pat. No.

0654 +b 5,244,797, +l in Kotewicz, M. L., et al., +i Nucl. Acids Res. +b <<<<
>>>>16:265 +l (+b 1988+l), in

0655 Gerard, G. F., et al., +i FOCUS +b 14+l (+b 5+l):+b 91 +l (+b 1992+l <<<<
>>>>), and in U.S. Pat. No.

0656 +b 5,668,005, +l the disclosures of all of which are fully incorporat<<<<
>>>>ed herein by

0657 reference.

0658 +p Polypeptides having reverse transcriptase activity for use in the

0659 invention may be obtained commercially, for example from Life

0660 Technologies, Inc. (Rockville, Md.), Pharmacia (Piscataway, N.J.),

0661 Sigma (Saint Louis, Mo.) or Boehringer Mannheim Biochemicals

0662 (Indianapolis, Ind.). Alternatively, polypeptides having reverse

0663 transcriptase activity may be isolated from their natural viral or <<<<
>>>>bacterial

0664 sources according to standard procedures for isolating and purifying <<<<
>>>>natural

0665 proteins that are well-known to one of ordinary skill in the art (see,<<<<
>>>> e.g., Houts,

0666 G. E., et al., +i J. Virol. +b 29:517 +l (+b 1979+l)). In addition, <<<<
>>>>the polypeptides having

0667 reverse transcriptase activity may be prepared by recombinant DNA

0668 techniques that are familiar to one of ordinary skill in the art (see,<<<<

>>>> e.g.,

0669 Kotewicz, M. L., et al., +i Nucl. Acids Res. +b 16:265 +l (+b 1988+l <<<<
>>>>); Soltis, D. A., and

0670 Skalka, A. M., +i Proc. Natl. Acad. Sci. USA +b 85:3372+14 3376 +l <<<<
>>>>(+b 1988+l)).

0671 +p Preferred polypeptides having reverse transcriptase activity for <<<<
>>>>use in

0672 the invention include M-MLV reverse transcriptase, RSV reverse

0673 transcriptase, AMV reverse transcriptase, Rous Associated Virus (RAV)

0674 reverse transcriptase, Myeloblastosis Associated Virus (MAV) reverse

0675 transcriptase and Human Immunodeficiency Virus (HIV) reverse <<<<
>>>>transcriptase,

0676 and others described in WO +b 98/47921 +l and derivatives, variants, <<<<
>>>>fragments or

0677 mutants thereof, and combinations thereof. In a further preferred <<<<
>>>>embodiment,

0678 the reverse transcriptases are reduced or substantially reduced in <<<<
>>>>RNase H

0679 +pg,24

0680 activity, and are most preferably selected from the group consisting <<<<

>>>>of M-MLV

0681 H+hu +31 +l +0 reverse transcriptase, RSV H+hu +31 +l +0 reverse <<<<

>>>>transcriptase, AMV H+hu +31 +l +0 reverse

0682 transcriptase, RAV H+hu +31 +l +0 reverse transcriptase, MAV H+hu +31 <<<<

>>>>+l +0 reverse transcriptase and

0683 HIV H+hu +31 +l +0 reverse transcriptase, and derivatives, variants, <<<<

>>>>fragments or mutants

0684 thereof, and combinations thereof. Reverse transcriptases of <<<<

>>>>particular interest

0685 include AMV RT and M-MLV RT, and more preferably AMV RT and M-MLV

0686 RT having reduced or substantially reduced RNase H activity

0687 (preferably AMV RT +60 H+hu +31 +l /BH+hu +30 +l +0 and M-MLV RT H+hu <<<<

>>>>+31 +l). The most preferred

0688 reverse transcriptases for use in the invention include SuperScript+198 ,

0689 SuperScript+198 +0 II, ThermoScript+198 +0 and ThermoScript+198 +0 II <<<<

>>>>available from Life

0690 Technologies, Inc. See generally, WO +b 98/47921, +l U.S. Pat. Nos. <<<<

>>>>+b 5,244,797 +l and

0691 +b 5,668,005, +l the entire contents of each of which are herein <<<<

>>>>incorporated by

0692 reference.

0693 +p A variety of DNA polymerases are useful in accordance with the

0694 present invention. Such polymerases include, but are not limited to, <<<<

>>>>+i Thermus

0695 thermophilus +l (Tth) DNA polymerase, +i Thermus aquaticus +l (Taq) DNA

0696 polymerase, +i Thermotoga neapolitana +l (Tne) DNA polymerase, +i <<<<

>>>>Thermotoga

0697 maritima +l (Tma) DNA polymerase, +i Thermococcus litoralis +l (Tli <<<<
>>>>or VENT+198)
0698 DNA polymerase, +i Pyrococcus furiosis +l (Pfu) DNA polymerase, <<<<
>>>>DEEPVENT+198 +0
0699 DNA polymerase, +i Pyrococcus woosii +l (Pwo) DNA polymerase, +i Bacillus
0700 sterothermophilus +l (Bst) DNA polymerase, +i Bacillus caldophilus +l <<<<
>>>>(Bca) DNA
0701 polymerase, +i Sulfolobus acidocaldarius +l (Sac) DNA polymerase,
0702 +i Thermoplasma acidophilum +l (Tac) DNA polymerase, +i Thermus <<<<
>>>>flavus +l (Tfl/Tub)
0703 DNA polymerase, +i Thermus ruber +l (Tru) DNA polymerase, +i Thermus
0704 brockianus +l (DYNAZYME+198) DNA polymerase, +i Methanobacterium
0705 thermoautotrophicum +l (Mth) DNA polymerase, Mycobacterium spp. DNA
0706 polymerase (Mtb, Mlep), and mutants, variants and derivatives thereof.
0707 +p DNA polymerases used in accordance with the invention may be any
0708 enzyme that can synthesize a DNA molecule from a nucleic acid template,
0709 typically in the +b 5+l +40 +0 to +b 3+l +40 +0 direction. Such <<<<
>>>>polymerases may be mesophilic or

0710 +pg,25

0711 thermophilic. Mesophilic polymerases include T+b 4 +l DNA polymerase, <<<<
>>>>T+b 5 +l DNA

0712 polymerase, T+b 7 +l DNA polymerase, Kienow fragment DNA polymerase, DNA

0713 polymerase III, DNA polymerase I and the like. Thermostable DNA

0714 polymerases include Taq, Tne, Tma, Pfu, VENT+198 , DEEPVENT+198 , Tth and

0715 mutants, variants and derivatives thereof (U.S. Pat. No. +b 5,436,<<<<
>>>>149; +l U.S.

0716 Pat. No. +b 5,512,462; +l WO +b 92/06188; +l WO +b 92/06200; +l WO +b <<<<
>>>>96/10640; +l Barnes,

0717 W. M., +i Gene +b 112:29+14 35 +l (+b 1992+l); Lawyer, F. C., et al.,<<<<
>>>> +i PCR Meth. Appl. +b 2:275+14

0718 287 +l (+b 1993+l); Flaman, J. -M., et al., +i Nucl. Acids Res. +b <<<<
>>>>22+l (+b 15+l):+b 3259+14 3260 +l (+b 1994+l)).

0719 +p DNA polymerases for use in the invention may be obtained

0720 commercially, for example from Life Technologies, Inc. (Rockville,

0721 Md.), Perkin-Elmer (Branchburg, N.J.), New England BioLabs

0722 (Beverly, Mass.) or Boehringer Mannheim Biochemicals

0723 (Indianapolis, Ind.).

0724 +p The present invention is also directed to nucleic acid molecules

0725 produced by the methods of the invention, which may be cDNA molecules,

0726 especially full-length cDNA molecules, to vectors (particularly <<<<
>>>>expression

0727 vectors) comprising these nucleic acid molecules and cDNA molecules <<<<
>>>>and to

0728 host cells comprising these nucleic acid molecules, cDNA molecules, <<<<
>>>>and/or

0729 vectors.

0730 +p Recombinant vectors may be produced according to this aspect of the

0731 invention by inserting, using methods that are well-known in the art, <<<<
>>>>one or
0732 more of the cDNA molecules or nucleic acid molecules prepared <<<<
>>>>according to
0733 the present methods into one or more vectors. The vector used in this <<<<
>>>>aspect
0734 of the invention may be, for example, a phage or a plasmid vector, and is
0735 preferably a plasmid. Preferred are vectors comprising cis-acting control
0736 regions to the nucleic acid encoding the polypeptide of interest. <<<<
>>>>Appropriate
0737 trans-acting factors may be supplied by the host, supplied by a <<<<
>>>>complementing
0738 vector or supplied by the vector itself upon introduction into the host.
0739 +p Expression vectors useful in the present invention include
0740 chromosomal-, episomal- and virus-derived vectors, e.g., vectors <<<<
>>>>derived from

0741 +pg,26

0742 bacterial plasmids or bacteriophages, and vectors derived from <<<<
>>>>combinations

0743 thereof, such as cosmids and phagemids, and will preferably include <<<<
>>>>at least

0744 one selectable marker such as a tetracycline or ampicillin resistance <<<<
>>>>gene for

0745 culturing in a bacterial host cell. Prior to insertion into such an <<<<
>>>>expression

0746 vector, the cDNA or nucleic acid molecules of the invention should be

0747 operatively linked to an appropriate promoter.

0748 +p Among vectors preferred for use in the present invention include

0749 pQE+b 70, +l pQE+b 60 +l and pQE-+b 9, +l available from Qiagen; pBS <<<<
>>>>vectors, Phagescript

0750 vectors, Bluescript vectors, pNH+b 8+l A, pNH+b 16+l a, pNH+b 18+l A, <<<<
>>>>pNH+b 46+l A, available

0751 from Stratagene; pcDNA+b 3 +l available from Invitrogen; pGEX, pTrxfus,

0752 pTrc+b 99+l a, pET-+b 5, +l pET-+b 9, +l pKK+b 223+l -+b 3, +l pKK+b <<<<
>>>>233+l -+b 3, +l pDR+b 540, +l pRIT+b 5 +l available

0753 from Pharmacia; and pSPORT+b 1, +l pSPORT+b 2, +l pSV.SPORT+b 1, +l <<<<
>>>>pCMVSPORT+b 6 +l

0754 and pCMVSPORT available from Life Technologies, Inc. Other suitable

0755 vectors will be readily apparent to the skilled artisan.

0756 +p The invention may be used in conjunction with any methods of cDNA

0757 synthesis that are well-known in the art (see, e.g., Gubler, U., and <<<<
>>>>Hoffman,

0758 B. J., +i Gene +b 25:263+14 269 +l (+b 1983+l); Krug, M. S., and <<<<
>>>>Berger, S. L., +i Meth. Enzymol.

0759 +b 152:316+14 325 +l (+b 1987+l); Sambrook, J., et al., +i Molecular <<<<

>>>>Cloning: A Laboratory

0760 Manual, +b 2+hu nd +l ed., Cold Spring Harbor, N.Y.: Cold Spring <<<<

>>>>Harbor Laboratory

0761 Press, pp. +b 8.60+14 8.63 +l (+b 1989+l); PCT US+b 98/19948; +l and <<<<

>>>>WO +b 98/51699+l) to produce

0762 cDNA molecules or libraries. Other methods of cDNA synthesis which may

0763 advantageously use the present invention will be readily apparent to <<<<

>>>>one of

0764 ordinary skill in the art.

0765 +p Having obtained cDNA molecules or libraries according to the present

0766 methods, these cDNAs may be isolated for further analysis or <<<<

>>>>manipulation.

0767 Detailed methodologies for purification of cDNAs are taught in the

0768 GENETRAPPER+198 +0 manual (Life Technologies), which is incorporated <<<<

>>>>herein

0769 by reference in its entirety, although alternative standard <<<<

>>>>techniques that are

0770 known in the art (see, e.g., Sambrook, J., et al., +i Molecular <<<<

>>>>Cloning: A

0771 Laboratory Manual, +b 2+hu nd +l ed., Cold Spring Harbor, N.Y.: Cold <<<<

>>>>Spring Harbor

0772 +pg,27

0773 Laboratory Press, pp. +b 8.60+14 8.63 +l (+b 1989+l)) may also be <<<<
>>>>used. The cDNA

0774 molecules or libraries produced by the invention may also be further

0775 manipulated by standard molecular biology techniques such as two hybrid

0776 analysis, cDNA normalization, sequencing and amplification. More

0777 particularly, the methods of the invention and the cDNA molecules or <<<<
>>>>libraries

0778 produced by such methods may be used in combination with RT-PCR and <<<<
>>>>+b 5+l +40 +0

0779 RACE technology (Life Technologies, Inc.) and differential display.

0780 +p A variety of inhibitors and binding molecules are suitable for use <<<<
>>>>in the

0781 present methods. Included among these inhibitors or binding molecules are

0782 antibodies that bind to the above-described polypeptides having reverse

0783 transcriptase activity (such as anti-RT antibodies including anti-AMV RT

0784 antibodies, anti-M-MLV RT antibodies or anti-RSV RT antibodies) or to cap

0785 structure (e.g., anti-cap antibodies), and fragments thereof (such as <<<<
>>>>Fab or

0786 F(ab+40)+hd 2 +l fragments). Such antibodies may be polyclonal or <<<<
>>>>monoclonal, and

0787 may be prepared in a variety of species according to methods that are <<<<
>>>>well-known

0788 in the art. See, for instance, Sutcliffe, J. G., et al., +i Science

0789 +b 219:660+14 666 +l (+b 1983+l); Wilson et al., +i Cell +b 37: 767 <<<<
>>>>+l (+b 1984+l); and Bittle, F. J., et al.,

0790 +i J. Gen. Virol. +b 66:2347+14 2354 +l (+b 1985+l). Antibodies <<<<
>>>>specific for any of the above-described

0791 reverse transcriptases or cap structures can be raised against the

0792 intact polymerase polypeptide or cap structures or one or more fragments

0793 thereof. These polypeptides or cap structures or fragments thereof may be

0794 presented together with a carrier protein (e.g., albumin) to an <<<<

>>>>animal system

0795 (such as rabbit or mouse) or, if they are long enough (at least about <<<<

>>>>+b 25 +l amino

0796 acids), without a carrier.

0797 +p As used herein, the term +37 antibody+38 +0 (Ab) may be used <<<<

>>>>interchangeably

0798 with the terms +37 polyclonal antibody+38 +0 or +37 monoclonal <<<<

>>>>antibody+38 +0 (mAb), except

0799 in specific contexts as described below. These terms, as used herein, are

0800 meant to include intact molecules as well as antibody fragments (such <<<<

>>>>as, for

0801 example, Fab and F(ab+40)+hd 2 +l fragments) which are capable of <<<<

>>>>specifically binding

0802 +pg,28

0803 to a polypeptide having reverse transcriptase activity (such as a DNA

0804 polymerase or a reverse transcriptase) or cap structures or portions <<<<

>>>>thereof.

0805 +p The antibodies used in the methods of the present invention may be

0806 polyclonal or monoclonal, and may be prepared by any of a variety of <<<<

>>>>methods

0807 (see, e.g., U.S. Pat. No. +b 5,587,287+l). For example, polyclonal <<<<

>>>>antibodies

0808 may be made by immunizing an animal with one or more polypeptides having

0809 reverse transcriptase activity or cap structures or portions thereof <<<<

>>>>according to

0810 standard techniques (see, e.g., Harlow, E., and Lane, D., +i <<<<

>>>>Antibodies: A

0811 Laboratory Manual, +l Cold Spring Harbor, N.Y.: Cold Spring Harbor

0812 Laboratory Press (+b 1988+l); Kaufman, P. B., et al., In: +i <<<<

>>>>Handbook of Molecular

0813 and Cellular Methods in Biology and Medicine, +l Boca Raton, Fla.: CRC

0814 Press, pp. +b 468+14 469 +l (+b 1995+l)). Alternatively, monoclonal <<<<

>>>>antibodies (or

0815 fragments thereof) to be used in the present methods may be prepared <<<<

>>>>using

0816 hybridoma technology that is well-known in the art (Kohler et al., +i <<<<

>>>>Nature

0817 +b 256:495 +l (+b 1975+l); Kohler et al., +i Eur. J. Immunol. +b <<<<

>>>>6:511 +l (+b 1976+l); Kohler et al.,

0818 +i Eur. J. Immunol. +b 6:292 +l (+b 1976+l); Hammerling et al., In: <<<<

>>>>+i Monoclonal Antibodies

0819 and T+l -+i Cell Hybridomas, +l New York: Elsevier, pp. +b 563+14 681 <<<<

>>>>+l (+b 1981+l); Kaufman,

0820 P. B., et al., In: +i Handbook of Molecular and Cellular Methods in <<<<

>>>>Biology and

0821 Medicine, +l Boca Raton, Fla.: CRC Press, pp. +b 444+14 467 +l (+b <<<<

>>>>1995+l)).

0822 +p It will be appreciated that Fab, F(ab+40)+hd 2 +l and other <<<<

>>>>fragments of the

0823 above-described antibodies may be used in the methods described herein.

0824 Such fragments are typically produced by proteolytic cleavage, using <<<<

>>>>enzymes

0825 such as papain (to produce Fab fragments) or pepsin (to produce <<<<

>>>>F(ab+40)+hd 2 +l

0826 fragments). Antibody fragments may also be produced through the <<<<

>>>>application

0827 of recombinant DNA technology or through synthetic chemistry.

0828 +p The invention also provides kits for use in accordance with the

0829 invention. Such kits comprise a carrier means, such as a box or <<<<

>>>>carton, having

0830 in close confinement therein one or more container means, such as <<<<

>>>>vials, tubes,

0831 bottles and the like, wherein the kit may comprise (in the same or <<<<

>>>>separate

0832 containers) one or more host cells, one or more reverse transcriptase<<<<

>>>>s, one or

0833 +pg,29

0834 more reverse transcription inhibitors, one or more cap binding <<<<
>>>>molecules, one

0835 or more DNA polymerases, suitable buffers, one or more nucleotides and/or

0836 one or more primers (e.g., oligo(dT) for reverse transcription). The kits

0837 encompassed by this aspect of the present invention may further comprise

0838 additional reagents and compounds necessary for carrying out standard <<<<
>>>>nucleic

0839 acid reverse transcription protocols.

0840 +p It will be readily apparent to one of ordinary skill in the <<<<
>>>>relevant art

0841 that other suitable modifications and adaptations to the methods and

0842 applications described herein are obvious and may be made without <<<<
>>>>departing

0843 from the scope of the invention or any embodiment thereof. Having now

0844 described the present invention in detail, the same will be more clearly

0845 understood by reference to the following examples, which are included

0846 herewith for purposes of illustration only and are not intended to be <<<<
>>>>limiting of

0847 the invention.

0848 +cl EXAMPLES

0849 +cl Example +b 1

0850 +cl Comparison of First Strand cDNA Synthesis With Varying Ratios of <<<<
>>>>Oligo (dT)

0851 Primer/mRNA

0852 +p This example compares first strand cDNA synthesis of the MAP+b 4 <<<<
>>>>+l gene

0853 with various ratios of oligo dT primer/starting mRNA. All components are

0854 available from Life Technologies, Inc., Rockville, Md., unless specified

0855 otherwise.

0856 +p The master mix for Superscript II reverse transcriptase (SS II RT) was

0857 prepared as specified in Table +b 1 +l below

0858 +pg,30

0859 +t,0300

0860 +p The master mix for ThermoScript+198 +0 II RT (TS RT) (AMV RT +60 <<<<
>>>>H+hu +31 +l +62 H+hu +30 +l)

0861 (see WO +b 98/47921+l) was prepared as specified in Table +b 2 +l below.

0862 +t,0301

0863 +p The master annealing mix was prepared by adding a +b 5 +l Kb MAP+b <<<<
>>>>4 +l

0864 mRNA, oligo(dT)+hd 25-30 +l and water to +b 5 +l tubes in the amounts <<<<
>>>>specified in Table +b 3

0865 +l below.

0866 +t,0302

0867 +pg,31

0868 +p The mixture was heated at +b 70+l +20 +0 C. for +b 10 +l minutes <<<<
>>>>and then chilled on ice

0869 for +b 5 +l minutes.

0870 +p Synthesis of first strand cDNA was done by adding +b 9 +l +82 l of the

0871 appropriate reverse transcriptase master mix, +b 10 +l +82 l of the <<<<
>>>>master annealing

0872 mix and +b 1+l +82 l of either SS II RT (+b 200 +l units/ul) or TS II <<<<
>>>>RT (+b 15 +l units/ul) for a

0873 total volume of +b 20 +l +82 l as summarized in Table +b 4 +l below.

0874 +t,0310

0875 +p The reactions were incubated for +b 1 +l hour at +b 45+l +20 +0 C. <<<<
>>>>for SS II RT and at +b 45,

0876 50 +l or +b 55+l +20 +0 C. for TS II RT. The tubes were placed on ice <<<<
>>>>to complete the

0877 reaction. +b 18 +l +82 l first stand cDNA of the reaction tube was <<<<
>>>>precipitated and re-suspended

0878 in +b 10 +l +82 l of water. +b 5 +l +82 l of the first strand cDNA <<<<
>>>>was mixed with +b 5 +l

0879 +82 l of standard loading buffer (+b 60 +l mM NaOH, +b 4 +l mM EDTA, <<<<
>>>>+b 0.1+l %

0880 +pg,32

0881 bromophenol blue), and loaded onto +b 1.4+l % alkaline agarose gel <<<<
>>>>for analysis.

0882 These results are shown in FIGS. 1 and 2.

0883 +p FIG. 1 is an autoradiograph of first strand cDNA synthesized with SS

0884 II RT at +b 45+l +20 +0 C. Lane M is the +b 1 +l kb DNA ladder. Lanes <<<<
>>>>+b 1+14 5 +l represents reaction

0885 conditions with a molar ratio of oligo(dT)+hd 25-30+l /mRNA of +b 1:1,<<<<
>>>> 2.5:1, 5:1, 10:1 +l

0886 and +b 50:1, +l respectively. FIG. 2 is an autoradiograph of first <<<<
>>>>strand cDNA

0887 synthesized with TS II RT. Lane M is the +b 1 +l kb DNA ladder. Lanes <<<<
>>>>+b 1+14 5 +l

0888 represent reaction conditions at +b 45+l +20 +0 C. with a molar ratio <<<<
>>>>of oligo(dT)+hd 25-30+l /mRNA

0889 of +b 1:1, 2.5:1, 5:1, 10:1 +l and +b 50:1, +l respectively. Lanes +b <<<<
>>>>6+14 10 +l represent

0890 reaction conditions at +b 50+l +20 +0 C. with a molar ratio of oligo <<<<
>>>>(dT)+hd 25-30+l /mRNA of +b 1:1,

0891 2.5:1, 5:1, 10:1 +l and +b 50:1, +l respectively. Lanes +b 11+14 15 <<<<
>>>>+l represent reaction

0892 conditions at +b 55+l +20 +0 C. with a molar ratio of oligo (dT)+hd <<<<
>>>>25-30+l /mRNA of +b 1:1, 2.5:1,

0893 5:1, 10:1 +l and +b 50:1, +l respectively. The results show that by <<<<
>>>>reducing the molar

0894 ratio of oligo(dT) primer/mRNA (preferably to +b 1:1+l) internal <<<<
>>>>priming with

0895 reverse transcriptase was almost entirely eliminated.

0896 +cl Example +b 2

0897 +cl Comparison of First Strand cDNA Synthesis Under Standard and Hot <<<<
>>>>Start
0898 Conditions
0899 +p This experiment was designed to compare first strand cDNA synthesis
0900 of the MAP+b 4 +l gene with standard reaction and hot start conditions.
0901 +p The annealing mix was prepared by mixing +b 1 +l +82 g of MAP+b 4 <<<<
>>>>+l mRNA and
0902 biotinylated Not I oligo(dT)+hd 25 +l primer ((Biotin)+hd 4 +l <<<<
>>>>GACTAGTTCTAGAT
0903 CGCGAGCGG CCGCCCTTTTT TTTTTTTTTTTT TTTTTTTT; see WO
0904 +b 98/51699+l) in the desired molar ratio of oligo (dT)/mRNA of +b <<<<
>>>>0:1, 1:1 +l or +b 15:1 +l in
0905 thin-walled PCR tubes and bringing the volume up to +b 10 +l +82 l <<<<
>>>>with water. If
0906 several tubes are identical, they may be made in one batch and aliquotted
0907 accordingly. The annealing mix was kept on ice.
0908 +p The master mix for Superscript II reverse transcriptase (SS II RT) was
0909 prepared as specified in Table +b 5 +l below.

0910 +pg,33

0911 +t,0330

0912 +p The SS II RT master mix was then divided into two equal aliquots, one

0913 for processing with standard reaction temperatures (batch +b 1+l) <<<<

>>>>and one for

0914 processing with hot start reaction temperatures (batch +b 2+l). To <<<<

>>>>allow for

0915 condensation, an additional +b 10+l % volume of water was added to <<<<

>>>>batch +b 2. +l All

0916 mixes were kept on ice.

0917 +p Synthesis of first strand cDNA was begun by briefly spinning tubes

0918 containing annealing mix to collect droplets, placing the tubes in a

0919 thermocycler and then heating them to +b 70+l +20 +0 C. for +b 10 +l <<<<

>>>>minutes. After this +b 10 +l

0920 minute cycle at +b 70+l +20 +0 C., the tubes of annealing mix for <<<<

>>>>batch +b 1 +l were immediately

0921 removed to ice. The tubes of annealing mix for batch +b 2 +l were <<<<

>>>>allowed to cool

0922 to +b 45+l +20 +0 C. in the thermocycler while the batch +b 2 +l <<<<

>>>>master mix was placed in the

0923 thermocycler and incubated at +b 45+l +20 +0 C. for +b 5 +l minutes. <<<<

>>>>After the +b 5 +l minute

0924 incubation, +b 11 +l +82 l of the master mix for batch +b 2 +l was <<<<

>>>>added to each batch +b 2 +l

0925 annealing tube and mixed with a pipette +b 2 +l times. Care was taken <<<<

>>>>not to spin

0926 the tubes to avoid lowering the temperature.

0927 +p +b 10 +l +82 l of the master mix for batch +b 1 +l was added to <<<<

>>>>each batch +b 1 +l

0928 annealing tube. The batch +b 1 +l tubes were lightly vortexed and briefly

0929 centrifuged to collect condensation droplets. The batch +b 1 +l tubes <<<<

>>>>were then

0930 returned to the thermocylcer and the tubes from both batch +b 1 +l <<<<

>>>>and +b 2 +l were

0931 incubated at +b 45+l +20 +0 C. for one hour.

0932 +p +b 5 +l +82 l of the first strand cDNA from each tube was mixed <<<<

>>>>with +b 5 +l +82 l of

0933 standard loading buffer (+b 60 +l mM NaOH, +b 4 +l mM EDTA, +b 0.1+l <<<<

>>>>% bromophenol

0934 +pg,34

0935 blue) and loaded onto +b 1.4+l % alkaline agarose gel for analysis. <<<<

>>>>The results are

0936 shown in FIG. 3.

0937 +p FIG. 3 is an autoradiograph of first strand cDNA synthesized with SS

0938 II RT. Lanes +b 1, 3 +l and +b 5 +l represents batch +b 1 +l reaction <<<<

>>>>conditions with a molar

0939 ratio of biotinylated oligo(dT)/mRNA of +b 0:1, 1:1 +l and +b 15:1, <<<<

>>>>+l respectively.

0940 Lanes +b 2, 4 +l and +b 6 +l represents batch +b 2 +l reaction <<<<

>>>>conditions with a molar ratio of

0941 biotinylated oligo(dT)/mRNA of +b 0:1, 1:1 +l and +b 15:1, +l <<<<

>>>>respectively.

0942 +p First strand cDNA was also synthesized with TS II RT using +b 15 <<<<

>>>>+l units

0943 of TS II RT per +82 g mRNA using a biotinylated oligo(dT)/mRNA ratio <<<<

>>>>of +b 1:1 +l

0944 and +b 15:1. +l The same protocol described above was followed, <<<<

>>>>except that the

0945 temperature was varied to +b 50+l +20 +0 C. The results are shown in <<<<

>>>>FIG. 4. FIG. 4

0946 is an autoradiograph of first strand cDNA synthesized with TS II RT. <<<<

>>>>Lane M

0947 is the +b 1 +l kb DNA ladder. Lanes +b 1 +l and +b 3 +l represent <<<<

>>>>reactions conditions using

0948 standard reaction temperatures at a +b 1:1 +l ratio and +b 15:1 +l <<<<

>>>>ratio, respectively.

0949 Lanes +b 2 +l and +b 4 +l represent hot start reactions conditions at <<<<

>>>>a +b 1:1 +l ratio and +b 15:1 +l

0950 ratio, respectively, as described above.

0951 +p The results indicated that by dropping the reaction temperature to the

0952 reverse transcriptase reaction temperature after denaturation of the <<<<

>>>>primer and

0953 mRNA mixture, the reaction was started directly and internal priming was

0954 avoided entirely.

0955 +cl Example +b 3

0956 +cl Synthesis of Double Strand cDNA by Controlling the Reaction <<<<

>>>>Temperature

0957 and the Concentration of Salt and RNase

0958 +p This example describes the synthesis of double stranded cDNA by

0959 controlling the reaction temperature and the concentration of salt <<<<

>>>>and different

0960 ribonuclease (RNases) during the treatment of the cDNA/mRNA hybrids after

0961 first strand cDNA synthesis.

0962 +p First strand cDNA was synthesized as described above in Example +b <<<<

>>>>2 +l

0963 and digested with either RNase I or RNase A as further described below.

0964 +pg,35

0965 +p RNase I digestion of first strand cDNA was done by re-suspending the

0966 first strand cDNA in +b 180 +l +82 l of water and +b 20 +l +82 l of <<<<

>>>>+b 10+l +33 +0 RNase I buffer (+b 100 +l

0967 mM Tris-HCl (pH +b 7.5+l), +b 50 +l mM EDTA, +b 2 +l M sodium <<<<

>>>>acetate). +b 2.5 +l units of

0968 RNase I (+b 1 +l unit/+82 g mRNA) were added and the mixture was <<<<

>>>>mixed well. The

0969 RNase I digestion mixture was incubated at +b 25+l +20 +0 C. for +b <<<<

>>>>30 +l minutes and extracted

0970 with phenol/chloroform once. The supernatant was precipitated with +b <<<<

>>>>1 +l +82 l of

0971 glycogen, +b 100 +l +82 l of ammonium acetate and +b 800 +l +82 l of <<<<

>>>>ethanol.

0972 +p RNase A digestion of first strand cDNA was done by re-suspending the

0973 first strand cDNA in +b 200 +l +82 l of digestion buffer (+b 10 +l mM <<<<

>>>>Tris-HCl (pH +b 7.5+l),

0974 +b 250 +l mM NaCl). +b 12.5 +l ng of RNase A (+b 5 +l ng/+82 g mRNA) <<<<

>>>>were added and the

0975 mixture was mixed well. The RNase A digestion mixture was incubated at

0976 +b 25+l +20 +0 C. for +b 30 +l minutes and extracted with phenol/chlo<<<<

>>>>roform once. The

0977 supernatant was precipitated with +b 1 +l +82 l of glycogen, +b 100 <<<<

>>>>+l +82 l of ammonium

0978 acetate and +b 800 +l +82 l of ethanol.

0979 +cl Example +b 4

0980 +cl Enrichment of the Full-length cDNA Clones With Cap-binding Proteins

0981 +p This example describes enrichment of full-length cDNA clones with

0982 the cap-binding protein eIF+b 4+l E.

0983 +p cDNA was prepared by precipitating the RNase I treated first strand

0984 cDNA described in Example +b 3 +l above and washing with +b 70+l % <<<<

>>>>ethanol. The

0985 resulting pellet was dried at room temperature for +b 5 +l minutes, <<<<

>>>>and re-suspended

0986 in +b 210 +l +82 l of +b 10 +l mM KPO+hd 4, +b 100 +l mM KCl, +b 2 +l <<<<

>>>>mM EDTA, +b 6 +l mM DTT and +b 5+l %

0987 glycerol. The cDNA was stored on ice.

0988 +p eIF+b 4+l E glutathione sepharose +b 4+l B beads were prepared by <<<<

>>>>first mixing

0989 glutathione sepharose +b 4+l B beads (Pharmacia, Sweden) well. To <<<<

>>>>prepare eIF+b 4+l E

0990 beads, a recombinant host cell expressing GST tagged eIF+b 4+l E <<<<

>>>>protein (the

0991 eIF+b 4+l E gene was cloned into a GST fusion vector to create a N-<<<<

>>>>terminal GST-eIF+b 4+l E fusion gene) was grown and the fusion protein <<<<

>>>>was purified by standard

0992 +pg,36

0993 techniques. Thus, the invention also relates to recombinant host cells

0994 expressing eIF+b 4+l E protein (particuarly as fusion proteins), to <<<<

>>>>vectors

0995 comprising the genes expressing such proteins or fusion proteins and <<<<

>>>>to the

0996 recombinant proteins or fusion proteins produced. In the present <<<<

>>>>invention any

0997 tag can be used (e.g., His Tag, GST tag, HA tag, Trx tag, etc.). Such <<<<

>>>>tags may

0998 be positioned at the carboxy and/or N-terminal region of the eIF+b <<<<

>>>>4+l E gene.

0999 +p The GST-eIF+b 4+l E fusion protein was complexed with sepharose +b <<<<

>>>>4+l B

1000 beads by glutathione coupling using gluthionine sepharose +b 4+l B beads

1001 (Pharmacia Biotech) following the manufacturers protocols. +b 200 +l <<<<

>>>>+82 l of the

1002 beads were transferred to a +b 1.5 +l ml microcentrifuge tube, <<<<

>>>>centrifuged for +b 1 +l

1003 second, and +b 75 +l +82 l of supernatant was removed. The beads were <<<<

>>>>washed twice

1004 with +b 1 +l ml of reaction buffer (+b 10 +l mM KPO+hd 4, +b 100 +l <<<<

>>>>mM KCl, +b 2 +l mM EDTA, +b 6 +l mM

1005 DTT and +b 5+l % glycerol), and re-suspended in +b 258 +l +82 l of <<<<

>>>>reaction buffer,

1006 followed by the addition of +b 42 +l +82 l (+b 18 +l pmoles/+82 l) of <<<<

>>>>eIF+b 4+l E protein (+b 600 +l

1007 pmoles/+b 100 +l +82 l beads). The mixture was mixed on a head to <<<<

>>>>head roller at +b 4+l +20 +0 C.

1008 for +b 30 +l minutes. The mixture was then centrifuged for +b 1 +l <<<<
>>>>second, and the
1009 supernatant was removed. The beads were washed twice with +b 1 +l ml <<<<
>>>>of reaction
1010 buffer and once with +b 1 +l ml of +b 25 +l +82 g/ml yeast tRNA in <<<<
>>>>reaction buffer. +b 20 +l +82 l of
1011 reaction buffer and +b 5 +l +82 g of yeast tRNA were then added to <<<<
>>>>the beads. +b 200 +l +82 l
1012 of RNase I treated cDNA was added to the beads, and the content was mixed
1013 on a roller at room temperature for +b 1 +l hour. After +b 1 +l hour, <<<<
>>>>the mixture was
1014 centrifuged for +b 1 +l second, and the supernatant was removed. The <<<<
>>>>beads were
1015 washed twice with +b 1 +l ml of reaction buffer and once with +b 1 +l <<<<
>>>>ml of +b 500 +l +82 M GDP
1016 in reaction buffer. The cDNA was eluted twice with +b 250 +l +82 l of <<<<
>>>>+b 500 +l +82 M GDP
1017 in reaction buffer. The eluted solutions were pooled and centrifuged <<<<
>>>>for +b 1 +l
1018 minute to remove the beads. The eluted cDNA was extracted twice with an
1019 equal volume of phenol/chloroform. The cDNA was divided into two tubes
1020 and precipitated with +b 1 +l +82 l of glycogen, +b 0.5 +l volume of <<<<
>>>>+b 7.5 +l M ammonium
1021 acetate and +b 2.5 +l volume of ethanol.

1022 +pg,37

1023 +cl Example +b 5

1024 +cl Evaluation of the cDNA Library

1025 +p To evaluate the quality of the cDNA libraries constructed with the

1026 above-described full-length methods, the MAP+b 4 +l gene (+b 5+14 6 <<<<

>>>>+l kb) and other genes

1027 was selected as the target genes. MAP+b 4 +l and other cDNA clones <<<<

>>>>were isolated

1028 from libraries constructed by standard methods well-known in the art (see

1029 SuperScript+198 +0 Plasmid Manual, Life Technologies, Inc.) and the <<<<

>>>>above-described

1030 full-length methods with +b 3+l +40 +0 and +b 5+l +40 +0 GeneTrapper <<<<

>>>>cDNA Positive

1031 Selection System (Life Technologies, Inc., Rockville, Md.). The

1032 positive clones were size analyzed by PCR. Tables +b 6 +l and +b 7 +l <<<<

>>>>below summarizes

1033 the results of the enrichment of full-length cDNA clones in human <<<<

>>>>fibroblast

1034 cDNA libraries constructed with methods well-known in the art <<<<

>>>>(control) and

1035 the full-length methods described above (full-length method).

1036 +t,0370

1037 +p The control library was constructed with SS II RT using known methods.

1038 +t,0371

1039 +pg,38

1040 +p These results show that the full-length methods described above

1041 yielded +22 +b 90+l % full-length cDNA clones with the +b 5+l +40 +0 <<<<

>>>>GeneTrapper system,

1042 compared to +21 +b 13+l % using standard methods. Furthermore, the <<<<

>>>>above-described

1043 full-length methods yielded +22 +b 37+l % full-length clones with the <<<<

>>>>+b 3+l +40 +0 GeneTrapper

1044 system, as compared to +21 +b 7+l % using standard methods.

1045 +cl Example +b 6

1046 +cl First Strand cDNA Synthesis, RNase I Digestion and eIF-+b 4+l E <<<<

>>>>Capture

1047 +p All conditions and parameters described above in Examples +b 2, 3 +l

1048 (RNase I) and +b 4 +l were followed, except for the following: +b 4 <<<<

>>>>+l reactions of +b 10 +l +82 g

1049 of human fibroblast cytoplasmic mRNA were used per reaction (see WO

1050 +b 98/45311+l); the biotinylated primer-adapter (Biotin)+hd 4+l

1051 -GACTAGTTCTAGATCGCGAGCGGCCGCCC(T)+hd 25 +l was used at a +b 1 :1 +l

1052 primer/mRNA molar ratio; TS II RT was used at +b 50+l +20 +0 C.; and <<<<

>>>>SS II RT was used

1053 at +b 45+l +20 +0 C. Table +b 8 +l below summarizes the first strand <<<<

>>>>cDNA and eIF-+b 4+l E capture

1054 results.

1055 +cl Example +b 7

1056 +cl Second Strand cDNA Synthesis

1057 +p Second strand cDNA was synthesized by first dissolving each of the

1058 four reaction pellets obtained in Example +b 6 +l above in +b 104 +l <<<<

>>>>+82 l of DEPC-treated

1059 water and then adding the following reagents to each reaction:

1060 +p1 +b 4 +l +82 l of +b 5+l +33 +0 First Strand Buffer*

1061 +p1 +b 30 +l +82 l of +b 5+l +33 +0 Second Strand Buffer*

1062 +p1 +b 2 +l +82 l of +b 0.1 +l M DTT

1063 +p1 +b 4 +l +82 l of +b 10 +l mM dNTPs

1064 +p1 +b 1 +l +82 l of +i E. coli +l DNA ligase (+b 10 +l units/+82 l)

1065 +p1 +b 1 +l +82 l of +i E. coli +l RNAse H (+b 2 +l units/+82 l)

+pg,39

+p1 +b 4 +l +82 l of +i E. coli +l DNA polymerase (+b 10 +l units/+82 l)

+ps see SuperScript Plasmid System manual (Life Technologies, Inc., <<<<

>>>>Rockville,

Md.).

+p These reactions mixtures were then incubated for +b 2 +l hours at <<<<

>>>>+b 16+l +20 +0 C. +b 2 +l +82 l

of T+b 4 +l DNA polymerase (+b 5 +l units/+82 l) was added and <<<<

>>>>incubation at +b 16+l +20 +0 C. was

continued for +b 5 +l more minutes.

+cl Example +b 8

+cl Streptavidin Bead Preparation

+p During the last +b 30 +l minutes of the +b 2 +l hour second strand <<<<

>>>>reaction

described in Example +b 7 +l above, streptavidin paramagnetic beads <<<<

>>>>were prepared

as follows.

+p Streptavidin paramagnetic beads (Seradyn) were gently mixed by

pipetting until the beads were completely re-suspended. +b 150 +l +82 <<<<

>>>>l of the mixed

beads were transferred to the bottom of a microcentrifuge tube for each

reaction. The tubes were inserted into a Magna-Sep Magnetic Particle

Separator (Life Technologies, Inc., Rockville, Md.) (the magnet)

and let sit for +b 2 +l minutes. While the tubes were in the magnet, <<<<

>>>>the supernatant

was removed by pipetting and +b 100 +l +82 l of TE buffer (+b 10 +l <<<<

>>>>mM Tris-HCl (pH +b 7.5+l),

+b 1 +l mM EDTA) was immediately added to the beads.

+p The tubes were then removed from the magnet and the beads were

1087 gently re-suspended by finger tapping or vortexing at the lowest <<<<
>>>>setting. The
1088 tubes were re-inserted into the magnet After +b 2 +l minutes, the <<<<
>>>>supernatant was
1089 removed, the beads were re-suspended in +b 160 +l +82 l of binding <<<<
>>>>buffer (+b 10 +l mM
1090 Tris-HCl (pH +b 7.5+l), +b 1 +l mM EDTA, +b 1 +l M NaCl) and the <<<<
>>>>tubes were placed into a
1091 microcentrifuge tube rack.

+pg,40

+cl Example +b 9

+cl Capture of the Double-stranded cDNA Library

+p After incubating the second strand reaction with T+b 4 +l DNA <<<<
>>>>polymerase
as described in Example +b 7 +l above, the reaction mixtures were <<<<
>>>>placed on ice and
+b 10 +l +82 l of +b 0.5 +l M EDTA was added. Then the cDNA library <<<<
>>>>was captured
according to the following procedure (see generally WO +b 98/51699+l).

+p The paramagnetic beads prepared according to Example +b 8 +l were
transferred to the second strand reaction mixture tubes and gently <<<<
>>>>mixed by
pipetting and the suspension was incubated for +b 60 +l minutes at room
temperature. The tubes were then inserted into the magnet. After +b 2 <<<<
>>>>+l minutes,
the supernatant was removed and discarded.

+p +b 100 +l +82 l of wash buffer (+b 10 +l mM Tris-HCl (pH +b 7.5+l <<<<
>>>>), +b 1 +l mM EDTA, +b 500 +l
mM NaCl) was added to the beads, the beads were re-suspended by finger
tapping or gently vortexing at the lowest setting and the tubes were <<<<
>>>>re-inserted
into the magnet for +b 2 +l minutes. The supernatant was removed and <<<<
>>>>discarded.
This washing step was repeated one more time and then +b 100 +l +82 l <<<<
>>>>of wash buffer
was added to the beads. The tubes were then again inserted into the <<<<
>>>>magnet
for +b 5 +l minutes.

1111 +cl Example +b 10

1112 +cl Not I Digestion

1113 +p After the +b 5 +l minute incubation described in the last step of <<<<
>>>>Example +b 9, +l

1114 the supernatant was removed and discarded from the paramagnetic beads and

1115 +b 41 +l +82 l of autoclaved, distilled water, +b 5 +l +82 l of REact <<<<
>>>>+b 3 +l buffer, +b 4 +l +82 l of Not I was

1116 added and the beads were mixed well by pipetting. The reaction was then

1117 incubated for +b 2 +l hours at +b 37+l +20 +0 C. The tubes were then <<<<
>>>>inserted into the magnet

1118 for +b 2 +l minutes and the supernatant containing the cDNA library <<<<
>>>>was transferred

1119 to fresh tubes.

1120 +pg,41

1121 +p +b 50 +l +82 l of phenol:chloroform:isoamyl alcohol (+b 25:24:1+l <<<<
>>>>) was added to the

1122 supernatant, the solution was vortexed thoroughly, and then <<<<
>>>>centrifuged at

1123 room temperature for +b 5 +l minutes at +b 14,000+l +33 +0 g. +b 45 <<<<
>>>>+l +82 l of the upper, aqueous

1124 layer was carefully removed and transferred to fresh microcentrifuge <<<<
>>>>tubes. +b 23 +l

1125 +82 l of +b 7.5 +l M ammonium acetate, +b 1 +l +82 l of glycogen (+b <<<<
>>>>20 +l +82 g) and +b 172 +l +82 l of ethanol

1126 (+31 +b 20+l +20 +0 C.) was added. The solution was mixed well and <<<<
>>>>stored on dry ice (or +31 +b

1127 70+l +20 +0 C. freezer) for +b 15 +l min.

1128 +p The ethanol solution was then centrifuged at +b 4+l +20 +0 C. for <<<<
>>>>+b 30 +l minutes at

1129 +b 14,000+l +33 +0 g. The supernatant was carefully removed from the <<<<
>>>>small pellets.

1130 +b 100 +l +82 l of +b 70+l % ethanol was added and the tubes were <<<<
>>>>centrifuged at room

1131 temperature for +b 2 +l minutes at +b 14,000+l +33 +0 g. The ethanol <<<<
>>>>was removed and the

1132 pellets were dried in a speed-vac for +b 2 +l minutes or until dry. <<<<
>>>>The pellets were

1133 then dissolved in +b 20 +l +82 l of TE buffer (+b 10 +l mM Tris-HCl <<<<
>>>>(pH +b 7.5+l), +b 0.1 +l mM

1134 EDTA). The final yield of cDNA was determined by the Cerenkov counts

1135 (see Table +b 8 +l below).

1136 +t,0410

1137 +cl Example +b 11

1138 +cl Ligation of cDNA to the Vector and Introduction into +i E. coli

1139 +p From +b 10 +l to +b 30 +l ng of the un-fractionated or size <<<<

>>>>fractionated (+24 +b 1.5 +l kb by

1140 low melting gel electrophoresis) cDNA was ligated into a vector

1141 pCMVSPORT +b 6 +l (Life Technologies, Inc.). This ligation was <<<<

>>>>introduced into

1142 +i E. coli +l by electroporation as described in the SuperScript <<<<

>>>>Plasmid System

+pg,42

manual (Life Technologies, Inc., Rockville, Md.), except that the cloning vector was pre-digested with Not I and Eco RV.

+p Sequence analysis of randomly selected clones from the cDNA library constructed (+b 304 +l clones) were analyzed by +b 5+l +40 +0 and +b <<<<>>>>3+l +40 +0 sequencing to determine the total percentage of full-length random clones in the cDNA library. Sequences were compared for homology with GeneBank sequences. The results are summarized in Table +b 9 +l below. Based on the results, <<<<>>>>approximately

+b 68+l % of the random clones were full-length (including known full-<<<<>>>>length clones and unknown full-length clones). Thus, approximately +b 17+l % <<<<>>>>unknown full-length clones were obtained from the human fibroblast cytoplasmic mRNA library.

+t,0420

+cl Example +b 12

+cl RNAse Assay

+p First strand cDNA was treated with RNase A at +b 1000 +l ng/+82 g <<<<>>>>mRNA in TE buffer (+b 10 +l mM Tris-HCl (pH +b 7.5+l), +b 1 +l mM EDTA) and <<<<>>>>RNase I +b 25 +l to +b 40 +l u/+82 g mRNA in TEN (+b 10 +l mM Tris-HCl (pH +b 7.5+l), +b 5 +l mM <<<<>>>>EDTA (pH +b 8.0+l), +b 200 +l mM Sodium Acetate) at +b 37+l +20 +0 C. essentially as described <<<<>>>>in Example +b 3. +l However, this treatment with large amounts of RNase at elevated <<<<>>>>temperatures

resulted in libraries containing very small average cDNA insert size <<<<
>>>>(about
+b 200 +1 bp). Therefore, a second strand cDNA assay was developed to <<<<
>>>>determine
the optimal amount of RNase needed.

+pg,43

+p First strand cDNA (radioactively labeled and non-radioactively labeled) was synthesized using HeLa mRNA at +b 500 +l ng of <<<<>>>>RNA/reaction. The first strand cDNA was precipitated with ethanol and dissolved in DEPC-<<<<>>>>treated water. The cold first strand cDNA was added to RNase buffer with different amounts of RNase. After incubation for +b 30 +l minutes at <<<<>>>>+b 25+l +20 +0 C., the treated cDNA was extracted with phenol:chloroform and precipitated with ethanol. The treated cDNA was dissolved in DEPC-treated water, a second strand cDNA reaction was performed with +hu 32+l P-dCTP plus and <<<<>>>>minus RNase H. The reaction was extracted with phenol:chloroform and precipitated with ethanol. Equal amounts of cpm was electrophoresed into a +b 1.4+l % <<<<>>>>alkaline-agarose gel. The results are shown in FIGS. 5 and 6.

+p FIG. 5 is an autoradiograph of second strand cDNA synthesized using different amounts of RNase A. Lane M is the +b 1 +l kb DNA <<<<>>>>ladder. Lane +b 1 +l represents untreated first strand cDNA. Lane +b 2 +l represents <<<<>>>>untreated second strand cDNA. Lanes +b 3, 5, 7 +l and +b 9 +l represent second strand <<<<>>>>cDNA synthesized without RNase H and with RNase A concentrations of +b 0, 1.25 +l ng, <<<<>>>>+b 2.5 +l ng and +b 5 +l ng, respectively. Lanes +b 4, 6, 8 +l and +b 10 +l represent second <<<<>>>>strand cDNA synthesized with RNase H and with RNase A at concentrations of +b 0, <<<<

>>>>1.25 +l ng,

1185 +b 2.5 +l ng and +b 5 +l ng, respectively.

1186 +p FIG. 6 is an autoradiograph of second strand cDNA synthesized

1187 using different amounts of RNase I. Lane M is the +b 1 +l kb DNA <<<<

>>>>ladder. Lane +b 1 +l

1188 represents untreated first strand cDNA. Lane +b 2 +l represents <<<<

>>>>untreated second

1189 strand cDNA. Lanes +b 3, 5, 7 +l and +b 9 +l represent second strand <<<<

>>>>cDNA synthesized

1190 without RNase H and with RNase I concentrations of +b 0, 0.5 +l u, +b <<<<

>>>>1.25 +l u and +b 2.5 +l

1191 u, respectively. Lanes +b 4, 6, 8 +l and +b 10 +l represent second <<<<

>>>>strand cDNA

1192 synthesized with RNase H and with RNase I at concentrations of +b 0, <<<<

>>>>0.5 +l u, +b 1.25 +l

1193 u and +b 2.5 +l u, respectively.

1194 +p These gel analysis demonstrated that a concentration of +b 1.25 +l <<<<

>>>>ng of

1195 RNAse A (see FIG. 5) or +b 0.5 +l units of RNAse I (see FIG. 6) may <<<<

>>>>be optimal

1196 to use with +b 500 +l ng of starting mRNA.

+pg,44

+cl Example +b 13

+cl Preparation of Antibodies Against Cap Structure

+p The antibody to cap was generated using m+b 7+l guanosnine-KLH as the antigen. +b 1200 +l hybridomas were plated and only +b 120 +l <<<<>>>>colonies were generated.

Of these only +b 6 +l colonies were positive for cap. After further <<<<>>>>analysis, +b 3 +l were determined to have the affinity required. The first screen ELISA <<<<>>>>consists of binding m+b 7+l guanosine-BSA to an ELISA plate, block with BSA, bind hybridoma supernatants, react with secondary antibody and determine positives via a calorimetric reaction with BCIP/NPT. The secondary screen included incubating appropriate dilutions of the hybridoma supernatan<<<<>>>>ts with either +b 0.1 +l mM m+b 7+l GTP, +b 0.1 +l mM cap analog M+hu 7+l <<<<>>>>G+hu 5+40 +l ppp+hu 5+40 +l G, +b 0.5 +l mM m+b 7+l guanosine or +b 0.5 +l mM GTP. The pretreated supernatant was <<<<>>>>then used in the standard ELISA procedure The GTP did not compete with the m+b 7+l guanosine-BSA whereas the m+b 7 +l versions all competed <<<<>>>>efficiently.

+p Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understand<<<<>>>>ing, it will be obvious to one of ordinary skill in the art that the same can be <<<<>>>>performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the <<<<

>>>>scope of

1217 the invention or any specific embodiment thereof, and that such <<<<

>>>>modifications

1218 or changes are intended to be encompassed within the scope of the <<<<

>>>>appended

1219 claims.

1220 +p All publications, patents and patent applications mentioned in this

1221 specification are indicative of the level of skill of those skilled <<<<

>>>>in the art to

1222 which this invention pertains, and are herein incorporated by <<<<

>>>>reference to the

1223 same extent as if each individual publication, patent or patent <<<<

>>>>application was

1224 specifically and individually indicated to be incorporated by reference.

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1225   +pg,45

1226   +t,0450
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+pg,46

+cm What is claimed is:

+cm 1. A method for synthesizing one or more cDNA molecules or a population of cDNA molecules, comprising mixing at least one mRNA <<<<>>>>template, poly A RNA template or population of such templates with at least one polypeptid<<<<>>>>e having reverse transcriptase activity and an inhibitor of the polypeptide having <<<<>>>>reverse transcriptase activity, under conditions that inhibit, prevent, reduce or substantially <<<<>>>>reduce the synthesis of non-specific cDNA products when compared to when said inhibitor is absent; and <<<<>>>>synthesizing one or more cDNA molecules or a population of cDNA molecules.

+cm 2. The method of claim 1, wherein said inhibitor is an antibody <<<<>>>>or antibody fragment.

+cm 3. The method of claim 2, wherein said antibody or antibody <<<<>>>>fragment is polyclonal or monoclonal.

+cm 4. The method of claim 1, wherein said inhibitor of reverse <<<<>>>>transcriptase activity prevents or inhibits reverse transcriptase activity at low temperatures.

+cm 5. The method of claim 1, wherein said polypeptide is a reverse <<<<>>>>transcriptase selected from the group consisting of M-MLV RT, RSV RT, AMV RT, RAV <<<<>>>>RT, MAV RT and HIV RT, and derivatives, fragments, mutations and variants thereof.

+pg,47

+cm 6. The method of claim 5, wherein said reverse transcriptase is <<<<
>>>>reduced or
substantially reduced in RNase H activity.

+cm 7. The method of claim 1, wherein said conditions comprise <<<<
>>>>annealing or
hybridizing one or more primers to said template at temperatures that <<<<
>>>>inhibit, prevent, reduce
or substantially reduce internal priming.

+cm 8. The method of claim 7, wherein said temperature is within in <<<<
>>>>the range of +b 10+14 90+20 +0 C.

+cm 9. The method of claim 7, wherein said temperature is within the <<<<
>>>>range of about
+b 20+14 75+20 +0 +l C.

+cm 10. The method of claim 7, wherein said temperature is within the <<<<
>>>>range of about
+b 45+14 65+20 +0 +l C.

+cm 11. The method of claim 1, wherein said conditions comprise the <<<<
>>>>use of a primer
to template ratio between +b 15:1 +l and +b 1:15.

+cm 12. The method of claim 11, wherein said primer to template ratio <<<<
>>>>is between +b 10:1 +l
and +b 1:10.

1260 +pg,48

1261 +cm 13. The method of claim 11, wherein said primer to template ratio <<<<
>>>>is between +b 5:1 +l

1262 and +b 1:5.

1263 +cm 14. The method of claim 1, wherein said conditions comprise the <<<<
>>>>use of a primer

1264 having a length of between +b 200 +l and +b 100 +l bases.

1265 +cm 15. The method of claim 14, wherein said length is between +b 20 <<<<
>>>>+l and +b 75 +l bases.

1266 +cm 16. The method of claim 14, wherein said length is between +b 20 <<<<
>>>>+l and +b 50 +l bases.

1267 +cm 17. The method of claim 14, wherein said length is between +b 25 <<<<
>>>>+l and +b 35 +l bases.

1268